

UNIVERSITY OF ALBERTA

OPTIMIZATION OF THERAPEUTIC MONITORING OF NEW
IMMUNOSUPPRESSIVE DRUGS:
CYCLOSPORIN G AND MYCOPHENOLATE MOFETIL

BY

LORALIE JEANETTE LANGMAN



A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**
IN

MEDICAL SCIENCES-
LABORATORY MEDICINE AND PATHOLOGY

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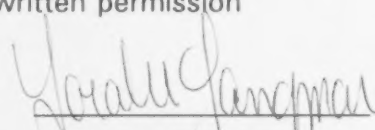
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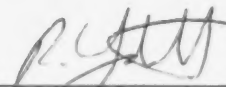
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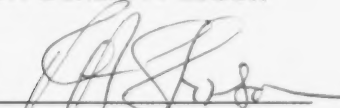
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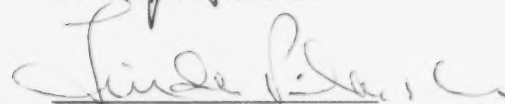
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DEDICATION

This thesis is dedicated to:

Z. Beeblebrox and Psychobunny

ABSTRACT

Therapeutic monitoring of immunosuppressive drugs is not only integral in patient management but in the development of new drugs and their assessment throughout clinical trials. Optimization of monitoring allows for more accurate assessment of the patient current status.

Cyclosporin G (CsG) and Mycophenolate Mofetil (MMF), two new immunosuppressive drugs, can be used to evaluate various factors required to determine appropriate monitoring of drug therapy. During clinical trials with CsG monitoring was performed using commercially available assays originally designed for the measurement of Cyclosporin A (CsA), since the cross-reactivity of CsG was significant in these assays. The cross-reactivity of the metabolites in the assays was minimal and comparable to what was found for CsA metabolites. The steady-state concentration of the metabolites in renal transplant recipients was assessed and found to contribute minimally to the observed biases for immunoassay versus HPLC. The *in vitro* toxicity of CsG and metabolites were comparable to CsA and its metabolites in the assay system used. From these data the clinical significance index for CsG and its metabolites was calculated. The results indicate that the former have values <10% of parent drug. Therefore assays selective for parent drug should be used for routine monitoring.

MMF is a pro-drug of mycophenolic acid (MPA), and has been recently approved for use in renal transplant recipients. Plasma was determined to be

the medium of choice for monitoring of MPA, since the majority of the drug (>99%) was found to be in this fraction. It had not been established if the measurement of plasma MPA or the measurement of the biological response to the drug (pharmacodynamic monitoring) best correlates to graft survival. The advantage of a pharmacodynamic monitoring approach is that it measures the total effect of a drug. This includes any contribution by biologically relevant metabolites.

A relatively simple pharmacodynamic assay was developed to measure the activity of the target enzyme of MPA, inosine monophosphate dehydrogenase (IMPDH). *In vitro* rabbits and humans have similar dose response curve with a concentration required for 50% inhibition of enzyme activity from 2-5 mg/L. In the canine model, however, the concentration required for 50% inhibition was >200 mg/L. *In vivo* in rabbits, dogs, and humans there is an inverse relationship between IMPDH activity and MPA concentration in the plasma. A significant relationship exists between MPA concentration and graft survival and the former with dose of MMF. There was a negative correlation between MPA concentration and IMPDH activity, while a trend to inverse relationship between graft survival and IMPDH activity was found. The data suggests the measurement of the biological response may provide a useful adjunct to traditional Therapeutic Drug Monitoring (TDM) for optimization of dosing of immunosuppressive drugs.

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I would also like to thank Pat Chackowski and Leslie Schmidt for their excellent technical assistance and friendship throughout my studies.

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I would like to thank the many people who have offered encouragement, guidance, and contributions to several research projects.

To my parents, Manson and Jeanette, I would like to thank you for your love, encouragement, and support throughout, and to my fiancé, Anthony, whose loving support and friendship have enabled me to achieve what I have.

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LIST OF ABBREVIATIONS

aa	amino acid
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ARRE-1	antigen receptor and response element-1
ARRE-2	antigen receptor and response element-2
AP-1	activating protein-1
ALP	alkaline phosphatase
APC	antigen presenting cell
AZA	azathioprine
Asp	aspartate
AUC	area under the curve
ACD	acid citrate dextrose
AUMC	area under the moment curve
Ca ²⁺	calcium
CD2	cluster designation 2
CD3	cluster designation 3
CD28	cluster designation 28
CsA	Cyclosporin A
CsG	Cyclosporin G (NVa ² Cyclosporin, OG37-325)
CsC	Cyclosporin C
CT	Cyclotrac SP-RIA
cm	centimetre
CMV	cytomegalovirus
CAM	calmodulin
CN	calcineurin
CNA	calcineurin A subunit
CNB	calcineurin B subunit
CO ₂	carbon dioxide

C2	carbon in position 2
C4	carbon at the 4 position
Cl	clearance
Ci	curie
cDNA	copy deoxyribonucleic acid
cys	cysteine
Cmax	concentration maximum
CV	coefficient of variation
C-13 NMR	carbon-13 nuclear magnetic resonance
Da	dalton
°C	degrees celsius
d	day
DAG	diacyl glycerol
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
DSG	deoxyspergualin
DTH	delayed type hypersensitivity
DMEM	Dulbecco's minimal essential medium
dpm	decays (disintegrations) per minute
EBV	Epstein Barr Virus
EMIT	enzyme multiplied immunoassay
EDTA	ethylenediaminetetraacetate
ET-1	endothelin-1
ETOH	ethanol
FPIA	fluorescence polarization immunoassay
FAB-MS	fast atom bombardment mass spectroscopy
fmol	femtomole
fg	femtogram
g	gram
xg	relative centrifugal force

>	greater than
GI	gastrointestinal
G1	growth phase-1
G2	growth phase-2
GTP	guanosine triphosphate
GMP	guanosine monophosphate
GDP	guanosine diphosphate
GVHD	graft versus host disease
GM-CSF	granulocyte macrophage stimulating factor
G-CSF	granulocyte stimulating factor
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HLA	human leucocyte antigen
HSA	human serum albumin
HSP	heat shock protein
HSV	herpes simplex virus
HPV	human papilloma virus
h	hour
HPLC	high performance (pressure) liquid chromatography
HGPRT	hypoxanthine- guanine phosphoribosyltransferase
H ₂ O	water
HBSS	Hanks's balanced salt solution
HCl	hydrogen chloride
HDL	high density lipoprotein
Hx	hypoxanthine
IC ₅₀	concentration resulting in 50% of function
IL-X	interleukin-X
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
IP ₃	inositol 1,4,5-triphosphate
IMP	inosine monophosphate
IP ₃ R	inositol 1,4,5-triphosphate receptor

Ino	inosine
IVC	inferior vena cava
IMPDH	inosine monophosphate dehydrogenase
IV	intravenous
KDa	kilodalton
Kcat	catalytic rate constant
KBr	potassium bromide
kg	kilogram
KOH	potassium hydroxide
LDL	low density lipoprotein
<	less than
6-MP	6-mercaptopurine
M-CSF	macrophage stimulating factor
MHC	major histocompatibility complex
MHCI	major histocompatibility complex class I
MHCII	major histocompatibility complex class II
MMF	mycophenolate mofetil
MPA	mycophenolic acid
mRNA	messenger ribonucleic acid
MPAG	mycophenolic acid glucuronide
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
μ L	microlitre
mL	millilitre
min	minute
mol	mole
μ M	micromole per litre (micromolar)
μ g	microgram
mg	milligram
MeOH	methanol
mM	millimolar

mm	millimetre
M	molar, mole/litre
μCi	microcurie
MRT	mean resonance time
NF-AT	nuclear factor of activated T-lymphocytes
NFkB	nuclear factor of immunoglobulin k-light chain in B-cells
NF-ATc	nuclear factor of activated T-lymphocytes cytoplasmic component
NF-ATn	nuclear factor of activated T-lymphocytes nuclear component
NF-AT1	nuclear factor of activated T-lymphocytes
NFIL-2a	nuclear factor IL-2a
NAD (NAD ⁺)	nicotinamide adenine dinucleotide oxidized form
NADH	nicotinamide adenine dinucleotide reduced form
nM	nanomolar
nm	nanometre
NA ₂ HPO ₄	sodium phosphate monobasic
NaCl	sodium chloride
O ₂	molecular oxygen
%	percent
6-keto-PGF _{1α}	6-keto-prostaglandin F _{1α}
PK	pharmacokinetic
PD	pharmacodynamic
PPIase	peptidyl-prolyl-cis-trans isomerase
PLC	phospholipase C gamma
PIP ₂	phosphatidyl-inositol-4,5-bisphosphate
PMA	phorbolmyristic acid
PKC	protein kinase C
PRPP	5'-phosphoribosyl-1-phosphate
PCA	perchloric acid

P-450	cytochrome P-450 system
P-450NF	cytochrome P-450 IIIa
PGI ₂	prostacyclin
pH	negative log of the hydrogen ion concentration
PO	oral administration
PHA	phytohemagglutinin
RAPA	rapamycin
RNA	ribonucleic acid
RIA	radioimmunoassay
RPMI	Roswell Park Memorial Institute (tissue culture media)
RBC	red blood cell
SS	Sandimmune specific immunoassay
NS	Sandimmune nonspecific immunoassay
SD	standard deviation
SE	standard error
SC	subcutaneous
SVC	superior vena cava
TAT-1	transactivator protein
TCR	T-cell receptor
TCA	trichloroacetic acid
TDM	therapeutic drug monitoring
TPMT	thiopurine methyltransferase
³ H	tritium
t _{1/2}	half life
BID	twice daily
TID	three time daily
U (IU)	international units
VD _{ss}	volume of distribution at steady state
VD	volume of distribution
VLDL	very low density lipoprotein
WB	whole blood

WBC

white blood cell

XMP

xanthine monophosphate

QUOTATION

The scientist takes off from the manifold observations of predecessors, and shows his intelligence, if any, by his ability to discriminate between the important and the negligible, by selecting here and there the significant steppingstones that will lead across the difficulties to new understanding. The one who places the last stone and steps across to the terra firma of accomplished discovery gets all the credit. Only the initiated know and honor those whose patient integrity and devotion to exact observation have made the last step possible.

As I Remember Him (1940), ch.20.
Hans Zinsser 1878-1940.

42

The Hitchhiker's Guide to the Galaxy. 1st Edition,
Harmony Books, New York, (1980)
Douglas Adams.

I. INTRODUCTION

1. HISTORICAL OVERVIEW OF IMMUNOSUPPRESSION

In 1908 Benjamin and Sluka observed that total body irradiation of rabbits impaired the synthesis of antibodies toward bovine serum (1). Six years later Murphy showed that irradiation slowed the development of immunity toward tumour allografts (1). Although total lymphoid irradiation did improve transplant outcome, there was a tendency toward opportunistic infections and, unpredictable, slowly reversible and potentially fatal outcomes (1).

The possibility of using "chemical" means to impair the immune system was investigated. In 1914 Murphy, and two years later Hekton, documented the immunosuppressive effects of the simple compounds benzene and toluene (1). In 1952 it was noted that the combination of methyl-bis-[β -chloroethyl]-amine hydrochloride (nitrogen mustard), corticosteroids, and splenectomy prolonged graft survival of mongrel canine allografts (1). Dameshek and Schwartz found that the anti-leukemic drug 6-mercaptopurine (6-MP) could prevent rabbits from producing antibodies to many antigenic stimuli (1). This initiated the modern era of pharmacologic immunosuppression. 6-MP is an analogue of the purine base hypoxanthine in which the 6-hydroxyl group has been replaced by a thiol group (2). During the breakdown of 6-MP, thioinosinic acid is produced. The latter competes with its analogue inosinic acid for the enzyme inosine monophosphate dehydrogenase, which converts inosinic acid

to xanthylic acid. This conversion is a critical step in the *de novo* synthesis of purine nucleotides. 6-MP was susceptible to gut hydrolysis, and in order to protect the unshielded mercapto group an imidazole derivative was synthesised. The imidazole derivative of 6-MP azathioprine (AZA), prolonged the survival of canine renal allografts. 6-MP and AZA are known as antimetabolites since they interfere with the metabolism of purine nucleotides, ultimately leading to decreased DNA synthesis and cell cycle arrest (1).

AZA was used in clinical transplantation, but, it was not very effective in humans until corticosteroids were added. Thus from 1966 to 1978 the conventional dual drug therapy became AZA and prednisone (2). Although this drug combination improved graft survival, the incidence of morbidity was still significant.

Woodruff and Anderson (1963) and Waksman et al. (1961) independently prepared biological antilymphocyte serum by injecting lymphocytes from one species into another, inducing antibodies in the serum of the injected animals (2). Initially the antibodies raised against human T-cells, were used to prevent graft rejection and to treat ongoing rejection. This led to the development of powerful polyclonal reagents of high immunosuppressive activity (2). Although a broad degree of T-cell inactivation confers heightened clinical efficacy, an additional component of non-specific immunodepression results from antibody cross-reactivity with epitopes on granulocytes and on monocyte/macrophages. Furthermore, the intense T-cell inhibition increases the

incidence of viral infection and lymphomas. Although improved diagnostic tools and antiviral prophylaxis appear to have decreased the seriousness of complications, there is little doubt that immunosuppressive regimens that include polyclonal anti-lymphocyte serum (ALS) increased the incidence of morbidity (1).

Immunosuppressive therapy has been greatly improved by the accidental discovery of Cyclosporin A (CsA) in the 1970's. Cyclosporin A (CsA) is a member of a group of unique cyclic peptides of fungal origin, many of which possess immunosuppressive properties (3-5). A strain of fungi imperfecti was isolated from a soil sample obtained from Hardinger Vidda in Northern Norway. This fungus was later identified as Tolypocladium inflatum Gams. A fermentation product from this fungus was later purified and identified as CsA and subsequently shown to possess antimicrobial and immunosuppressive activity (6). The mechanism of action of this drug will be discussed later. The clinical use of CsA has had a major impact on the field of organ transplantation, dramatically improving graft survival and decreasing morbidity over previous immunosuppressant regimes which included the use of compounds such as AZA or Prednisone (7,8). The first animal studies were undertaken in 1974 to investigate its immunosuppressive activity both *in vitro* and *in vivo*. These preliminary studies led to the development of CsA as a primary immunosuppressant. The first clinical trials were undertaken in 1978 using CsA to prevent allograft rejection in cadaveric kidney transplant recipients and

in the prevention of Graft versus Host Disease (GVHD) in bone marrow transplantation (9,10,11).

CsA has not only significantly improved the short- and long-term survival of allografts, but it has also mitigated the impact of factors such as HLA mismatching and pre-transplant blood transfusions (3). Thus, CsA is clearly established as the principal immunosuppressant in renal transplantation, where multi-centre studies have demonstrated its superiority over previous agents in terms of both efficacy and safety. CsA has also been used in a wide variety of other clinical disorders, some of which have an autoimmune etiology (12,3), including rheumatoid arthritis (13), diabetes mellitus (14), psoriasis (15), uveitis (16), and multiple sclerosis (12). In contrast, CsA has had limited success in treating diseases such as systemic lupus erythematosus and myasthenia gravis (3). Despite the revolutionary impact on the field of organ transplantation, the use of CsA is not without several side-effects, including neuro-, hepato-, and nephrotoxicity, which seriously hamper its clinical use (9,3,17-31).

These side-effects have led researchers to investigate new potentially less toxic immunosuppressive drugs. Currently many new drugs are being investigated for the prevention of graft rejection. In addition to CsA, Tolypocladium inflatum produces a large number of minor fermentation products of the same type. At least 25 of these natural cyclosporines have been isolated and their chemical composition determined (5). Over 700 semisynthetic analogues have been produced and tested in vitro, but none have

been shown to possess a greater pharmacologic activity than CsA. The analogue to undergo the most extensive clinical evaluation is the naturally occurring analogue CsG. *In vitro* and *in vivo* it has been shown to be as potent as CsA (5).

The carboxy-cyclic macrolide FK506, isolated from the actinomycete Streptomyces tsukubensis, has an effect similar to that of CsA (32). FK506 is probably more effective in some types of transplants, particularly liver and intestine; it is also nephrotoxic. In the attempt to maximise its immunosuppressive effects by using high doses, FK506 has displayed greater toxic complications and was found to exhibit a narrower therapeutic window (1).

Of greater interest is a structurally related compound, Rapamycin (RAPA), produced by Streptomyces hygroscopicus. RAPA acts through the inhibition of a distinct calcium independent pathway that mediates transduction of the lymphokine signal (33). Although the mechanism of RAPA is not well understood, the most recent studies suggest that the target may be lipid kinases (33). Intense interest in this compound is due to its synergistic action with CsA (1), potentially reducing the dosage of CsA and the incidence of side effects. Several new antimetabolites inhibiting the synthesis of DNA and DNA transcription are under investigation. These include Mycophenolate Mofetil (MMF), Brequinar, and possibly Leflunomide (34). MMF has been extensively evaluated in Phase III clinical trials and was shown to be a potent

immunosuppressive agent. MMF, Brequinar and Leflunomide inhibit DNA synthesis by targeting key enzymes involved in purine and pyrimidine nucleotide biosynthesis via the *de novo* pathway.

Another class of immunosuppressive agents inhibits antigen presentation or processing, such as OKT3, a monoclonal antibody, and chemotherapeutic agents like Deoxyspergualin (DSG). Monoclonal antibodies could be directed against cell surface markers on T-cells or on the antigen presenting cells (APC). The antibodies decrease recognition of foreign antigens by binding to molecules on T-cells that initiate or potentiate signal transduction leading to activation, such as the major Histocompatibility complex (MHC), T-cell receptor (TCR) or CD3 (35). DSG acts by an uncertain mechanism on T-cells (36,37). There are two possible explanations for the immunosuppressive action. First, DSG binds to heat shock proteins that may be involved in binding and intracellular transport of antigenic peptides within the APC, acting as a peptide mimic (36,37). The second explanation for DSG mechanism of action is that it may interfere with the interactions of heat shock proteins (HSP) that are required for proper activation of glucocorticoid receptors (37). However, DSG has shown limited clinical promise as a prophylactic agent in initial studies (1).

2. PROBLEMS WITH CURRENT IMMUNOSUPPRESSIVE AGENTS

Transplantation has become the treatment of choice in many situations, including end stage renal disease, providing significant medical and economic

advantages over dialysis (12). With the introduction of CsA to the traditional inductive immunosuppression regime of AZA and steroids, graft survival in primary recipients is in the range of 85 to 95% after 1 year following cadaveric or living related donor transplantation, with a subsequent attrition rate of 2 to 3% per year (12). This triple therapy regime of CsA, AZA, and prednisone is still more common than dual therapy composed of AZA and CsA. The incidence of acute rejection is decreased with the use of the former (2). The introduction of CsA has improved patient outcome, particularly in high risk groups such as the elderly, diabetic patients, and sensitized, repeatedly grafted individuals. Due to the depression of the immune system the recipient does not mount an immune response against the grafted organ. However, the impairment of the immune system may potentially cause opportunistic infections. Viral infections are common in patients, especially Epstein-Barr virus (EBV), Human Papilloma (HPV) and Herpes Simplex viruses (HSV). Bacterial and fungal infections are not uncommon. One of the main concerns is EBV; it causes B-cell proliferation, and has possible connections to lymphomagenesis (39). High doses of immunosuppressive drugs can also increase the incidence of tumours, since primary or acquired immunodeficiency is associated with an increased incidence of non-Hodgkins lymphomas and other rare tumours, including squamous cell carcinoma of the skin and Kaposi-Sarcoma (39). Impaired immune surveillance may result in the uncontrolled growth of a neoplastic cell arising by somatic mutation or viral infection (39). In addition

to the above mechanism some immunosuppressive agents may have a direct oncogenic action. AZA for example has been shown to cause chromosomal breaks that can lead to mutagenic events (39).

Immunosuppression has side effects as described above, however, the drugs themselves often have toxic effects. The major toxic effect is myelotoxicity, including leucopenia, thrombocytopenia, and anemia (40). Approximately 20% of kidney transplant patients experience leukopenic episodes, the frequency of which are related to the dose. Upon withdrawal of the drugs the bone marrow recovers quickly (40). However, in addition to the myelotoxicity associated with its antiproliferative effects, AZA has several limitations. Relatively high drug concentrations are required for effective immunosuppression due to its competitive mechanism of action, and its hepatotoxic effect (1,41).

In organ transplantation steroids are frequently administered in high concentrations as prophylaxis against rejection or for treatment of rejection after it has occurred. The side effects are numerous and include impaired wound healing, osteoporosis, obesity, hypertension, avascular necrosis, cataracts, diabetes, pancreatitis, psychiatric disorders, and impaired growth and development (40). In pediatric patients it is often necessary to give very large doses of steroids, which suppress growth and cause cushingoid changes (truncal obesity, "humped back", abnormal hair growth, and facial fat deposits) (2). Due to the side effects, steroids are often administered on alternate days

or are slowly tapered to a lower dose or discontinued entirely.

The final drug in the standard triple therapy regime for immunosuppressive drugs is CsA. Despite the significant impact on graft survival, the use of CsA is not without several side effects, many of which seriously hamper its clinical use (3,4,18-31). Neurological side effects, including tremors and seizures (4,18,20), are common in patients receiving CsA and affect over 20% of those receiving the drug (3,4). Other reported side effects include hirsutism (21), hyperuricemia, and a variety of gastrointestinal complications including stomach upset, nausea, and bloating (3). However, there is no direct toxic effect on the structure and function of the gastrointestinal mucosa. Infectious complications are observed more frequently with CsA than with other immunosuppressive regimes such as steroids (22). Furthermore, patients receiving CsA have an increased risk of developing malignant tumours (4). CsA also causes vascular complications including thrombosis, increased platelet aggregation, and alterations in prostaglandin production. All of which ultimately contribute to the hypertension (25).

Hepatotoxicity is a frequent side effect of CsA, affecting almost half of the patients who receive the drug (4,23,24). Biochemical alterations include increased serum bilirubin, alkaline phosphatase, γ -glutamyl transferase, aminotransferases, and bile acids (3,23). In contrast, there is decreased bile flow and a slight decrease in serum albumin. Morphologically, cholestasis, vacuolization, and cell necrosis of the hepatic parenchyma are seen (23).

Nephrotoxicity is the side effect of most concern, affecting virtually all patients who receive the drug (17). In humans at least three patterns have been observed: functional changes, structural changes, and irreversible chronic nephrotoxicity. CsA induced functional changes result in both tubular and vascular alterations (28-31). The functional changes are due to alterations in the reabsorption and excretion of compounds, leading to decreased serum magnesium and mild increases in potassium and uric acid (29,42). These changes are often seen with therapeutic doses of the drug and are reversible upon dose reduction. The functional changes occurring in the vasculature result from increased vasoconstriction of the afferent arterioles. This leads to decreased kidney perfusion, decreased filtration, and decreased glomerulofiltration rate (GFR), resulting in increased serum creatinine and urea, and possibly enzymeuria and aminoaciduria (29,43). These changes are also reversed upon dose reduction (28,29).

Structural changes to the renal tubules are confined to the proximal tubule and consist of vacuoles, giant mitochondria, single cell necrosis and microcalcification (29,30,44,45). These changes are reversible upon reduction of the dose. However, irreversible chronic nephrotoxicity is characterized by interstitial fibrosis and arteriolopathy (26,27,46). These are thought to arise from a CsA induced vasoconstriction of the afferent arteriole (17,28,30,47-50). These changes can lead to an increase in renal vascular resistance, a decrease in renal plasma flow, and glomerular hypofiltration. The latter may result in the

collapse of the associated glomerulus and interstitial fibrosis. These changes are irreversible and result in permanent damage to the kidney (30,31). There is evidence to suggest that the pathogenesis of CsA renal side-effects involves alterations in the release of vasoconstrictive substances and/or vasodilatory prostanoids (17,48). A critical balance between these substances is required in order to maintain vascular tone. Many of the functional and tubular changes are dose dependant and can be reversed or lessened by reducing the dosage. For this reason it is recommended that CsA levels be monitored to minimize the toxic effects while still maintaining adequate immunosuppression (3,17).

3. NEW IMMUNOSUPPRESSIVE DRUGS: CYCLOSPORIN G AND MYCOPHENOLATE MOFETIL

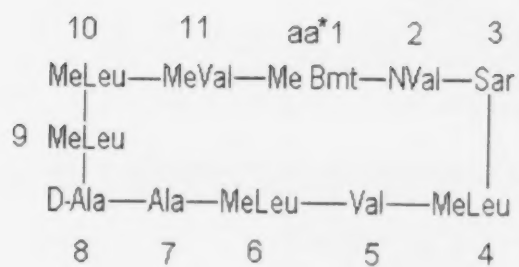
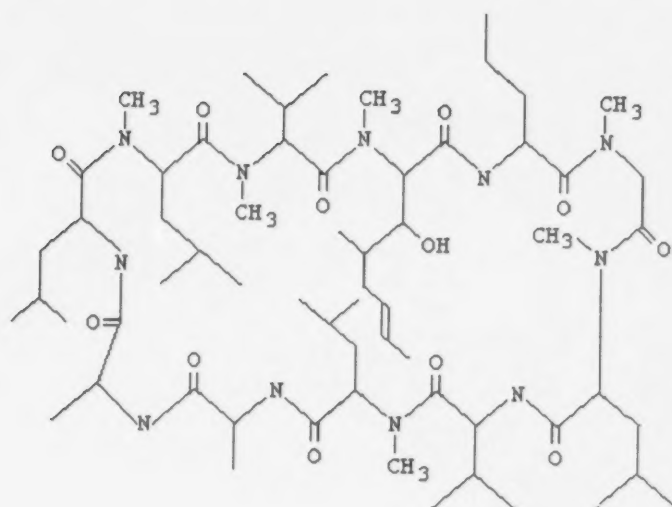
The traditional immunosuppressive therapy (CsA, AZA, steroids) has significant side-effects. These side-effects have led investigators to search for other immunosuppressive drugs in which their frequency are reduced, or when used in conjunction with CsA can reduce the dosage and consequently the side-effects (3,4). One approach was to evaluate the numerous analogues of CsA to identify ones that possess immunosuppressive activity but not toxicity. Studies of the relationship between the structure and biologic activity have shown that amino acids 1, 2, 3, 10, and 11 are important for the immunosuppressive properties of CsA (Figure I-1), and that the unique amino acid butyl-dimethyl-threonine (MeBmt) at the 1 position is essential for

biological activity (5). The analogues with strong immunological activity include CsC, D, G, M, val²-dihydrocyclosporine D, and dihydrocyclosporine C (5). Most of these analogues proved to be more toxic than CsA in either renal, hepatic, or neurological side-effects. However, studies in animals have indicated that CsG (NVa²-Cyclosporin) is as efficacious as CsA in the prevention of allograft rejection with similar or reduced frequency of renal side-effects (51-54). CsG differs from CsA at position 2 of the molecule where the alpha aminobutyric acid residue of the latter is replaced by norvaline (Figure I-1) (5). Previous studies have indicated that the structure and activity of CsG metabolites are similar to those of CsA (55). This suggests that both drugs are metabolized in a similar manner (56-58).

The immunosuppressive effect of CsG has been studied in a number of *in vitro* and *in vivo* models. *In vitro*, CsG has been shown to be as potent as CsA in the suppression of mixed lymphocyte cultures (MLC), cell-mediated lympholysis, and mitogen-induced cell proliferation (5). *In vivo* immunological studies have been shown CsA to be effective in the prevention of primary Delayed Type Hypersensitivity (DTH) reactions, and localized GVHD (5). The use in transplant models has produced some conflicting results. However, most of the studies found CsG to be as effective as CsA in the prevention of graft rejection (5). CsG has undergone extensive Phase 2 and 3 clinical trials in renal transplant recipients. No significant differences were reported in the incidence of rejection, patient-and-graft survival, infection, and hypertension

Figure I-1

Structure of Cyclosporin G (CsG)



*amino acid number

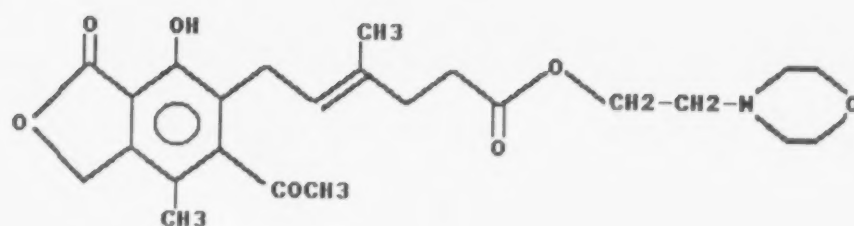
adapted from Simmons RL, Wang SC. Transplan Proc 1991;23:2152-2156.

between appropriately-matched patients receiving CsA or CsG (59-61). There did not appear to be a difference in the occurrence of nephrotoxicity between the two groups, nor was there a difference in the histological pattern of nephrotoxicity (61). Unfortunately, hepatotoxicity was associated with CsG treatment, as indicated by an elevation of bilirubin and liver enzymes (59-61). The improvement and normalization of bilirubin and liver enzymes after withdrawal of CsG points to a drug induced hepatotoxicity (59-61). In contrast, patients receiving CsG exhibited marginally improved renal function as assessed by creatinine and inulin clearance (59).

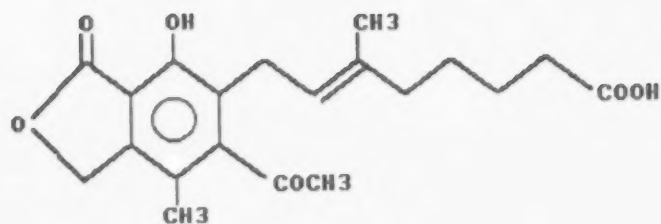
New drugs that could be used in conjunction with CsA have been investigated; one such drug is MMF. Hoa et al. have shown that MMF when given in conjunction with CsA can produce synergistic effects (62). Therefore, the administered dose of CsA could be lowered, thus reducing CsA associated side effects. MPA was first extracted from the fungus Penicillium brevicompactum. However, MPA is made by many species of the genus Penicillium (63). The compound was first identified in 1896, with the crystal structure being reported in 1972 (64,65). Mycophenolate mofetil is the morpholinoethyl ester of mycophenolic acid. Chemically it is 2-(4-morpholino)ethyl-(E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexanoate (Figure 1-2). MPA possesses antibacterial, antifungal, antiviral, antitumour properties and, more relevant to our studies, immunosuppressive properties (66). However, MPA failed to be

Figure I-2

Structure of Mycophenolate Mofetil (MMF) and Mycophenolic acid (MPA).



MYCOPHENOLATE MOFETIL



MYCOPHENOLIC ACID

adapted from Simmons RL, Wang SC. Transplant Proc 1991;23:2152-2156.

an effective agent in these other applications due to its potent immunosuppressive activity (66-69). Therefore, MPA was further developed as a new immunosuppressive agent for use in autoimmune diseases and for transplantation. MMF is rapidly hydrolysed by cytosolic esterase following absorption to yield the parent compound, MPA (70,71). The immunosuppressive activity of MMF is expressed only after the hydrolysis, since the terminal carboxyl group is necessary for activity (72,66).

Three multi-centre clinical trials in which MMF was evaluated for the prevention of acute rejection in cadaveric renal allograft recipients indicated that, at 6 months, biopsy proven rejection or treatment failures, as defined by graft loss, death, or premature withdrawal from the study for any reason, was decreased (73-75). Not only was the incidence of acute rejection or treatment failure significantly reduced in the MMF groups but the severity of rejection was lessened as well. This conclusion was based on the finding that MMF treated patients received fewer courses of anti-rejection therapy compared to the control group (73-75). In another study MMF was effectively used in the treatment of refractory renal allograft rejection (76). Nausea and diarrhea were the most common side effects and were usually self-limiting or resolved with dose reduction. Leukopenia occurred in few patients, who had concurrent CMV infections and were undergoing acyclovir (anti-viral) therapy. A similar study was undertaken for liver transplant recipients, again indicating its effectiveness in the treatment of refractory rejection (77).

MMF has proven to be efficacious in the prevention of early post-transplant rejection, using fixed doses of the drug (78,76). The toxic side effects are minimal; nephrotoxicity, bone marrow depression, and hepatotoxicity were not significantly noted (79,80). However, some researchers have reported a two fold increase in alkaline phosphatase (ALP) (81,82). It is believed that IMPDH inhibition results in increased levels of IMP. Since ALP is the enzyme responsible for the degradation of IMP, it is thought to be elevated in response to the accumulation of IMP (81,82).

4. MECHANISMS OF ACTION

i. T-cell activation

Activation of T-cells involves a complex cascade of biochemical events that ultimately result in induction of T-cell proliferation and differentiation. Activation begins with cell-cell interaction between an antigen presenting cell (APC) and the T-cell. T-cells are activated by peptide antigens presented in the folds of a class I or II major histocompatibility complex (MHC) on the surface of an APC (83). Specific T-cell activation requires a MHC II molecule combined with peptide antigen to bind with a T-cell receptor (TCR) specific for the peptide antigen; interactions with either antigen or MHC II alone are not recognized by the TCR. The TCR which has low affinity for the MHC II, does not directly participate in triggering signal transduction pathways. Instead, conformational changes are transduced to the cluster designation-3 (CD3)

resulting in initiation of intracellular second messenger signalling (84).

Although knowledge about the individual functional roles of each subunit of the TCR/CD3 complex is limited, it is speculated that the role of the $\alpha:\beta$ TCR is mainly recognition of an antigenic peptide fragment, while the role of the CD3 subunits centres around transmembrane signal transduction and initiation of intracellular second messenger systems (85,86). The TCR transmits the antigenic signal via conformational changes in the $\alpha:\beta$ (87). The signal is passed on to the CD3 components, through a coupling of the α of TCR, to the delta of the CD3. From this point the zeta-zeta is responsible for initiating intracellular signal pathways (88).

Once the TCR binds to the MHC II: antigen complex and transmembrane signal transduction is complete, intracellular events take over. In many systems, receptor mediated hydrolysis of phosphatidyl-inositol-4,5-bisphosphate (PIP_2) is a common mechanism for signal transduction of extracellular stimuli; this also seems to be the case with T-cells (89). PIP_2 is quickly broken down by phospholipase C gamma (PLC) to yield two second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). This ultimately results in release of Ca^{2+} into the intracellular space from Ca^{2+} storage sites and activation of protein kinase C (PKC).

Elevated Ca^{2+} concentrations favour the formation of complexes with the ubiquitous Ca^{2+} -dependant regulatory protein calmodulin (CAM). This leads to the activation of Ca^{2+} -CAM-dependant protein kinases, which have distinct

substrates from PKC (90). The phosphorylation of several different T-cell membrane and cytoplasmic proteins occurs within seconds following ligand binding of the TCR:CD3. The enzymes that may catalyze serine and threonine phosphorylation in T-cells include PKC and Ca^{2+} -CAM-dependant kinases such as calcineurin (CN) (90). CN (Protein phosphatase-IIB) is comprised of two subunits, a CAM binding 59 kDa catalytic subunit (CNA) and a Ca^{2+} binding 19 kDa regulatory subunit (CNB) (91). It is the key rate-limiting enzyme in the T-cell signal transduction cascade. The CN catalytic subunit consists of a catalytic domain in the N-terminal half of the protein, followed by allosteric control regions in the C-terminal third of the protein, a CAM binding domain and an autoinhibitory domain (91). CAM releases the autoinhibitory domain from the catalytic site of CN (91). CN activates the IL-2 promoter via at least three different transcription factors; nuclear factor of activated T-cells, (NF-AT), nuclear factor of immunoglobulin k light chain in B-cells (NF-kB), and Oct/OAP. Multiple Ca^{2+} sensitive signals are required to regulate IL-2 promoter activity (91). One model for CN's action in T-cells is that it dephosphorylates one or more transcription factors in the cytoplasm that can then be translocated to the nucleus to activate the IL-2 promoter. Calcineurin dephosphorylates a constitutive cytosolic component of the NF-AT complex (NF-ATc) which is translocated to the nucleus, and then combines with a nuclear component (NF-ATn), resulting in the NF-ATc/NF-ATn heterodimer, which binds to its regulatory site on the IL-2 promoter contributing to IL-2 expression (91). The

products of T-cell cytokine genes are significantly elevated within 4 hours of TCR:CD3-mediated stimulation.

The general structure of the cytokine gene consists of 4 or 5 exons extending over several kilobases of genomic DNA (92,93). The single copy genes are stable, and are unable to undergo rearrangements. The activation of some cytokine genes is co-ordinate; therefore, a reasonable assumption would be that certain regulatory elements, such as enhancer sequences and inductive mechanisms, are common (92,93). Several consensus sequences 8 to 18 nucleotides in length have been identified in the upstream regulatory regions of cytokine genes. Such regions located between 250 and 300 base pairs upstream of the start site of IL-2 and IL-2R genes have been identified as important regulatory sites.

The IL-2 genes have served as a paradigm for the regulation of cytokine gene transcription during T-cell activation. IL-2 is the autocrine growth factor for the majority of normal T-cells and, therefore, the transcription is essential for the mitotic component of functional T-cell activation. The transcription of the IL-2 gene begins within 1 h following TCR;CD3 mediated stimulation of normal human lymphocytes. The elevation in RNA transcripts of cytokine genes is largely due to increased transcription as opposed to decreased RNA degradation (90).

Several DNA binding proteins that bind to the IL-2 gene enhancer region are believed to be important links. Some sequences in this region bind a

transcription-activating protein complex called AP-1. These consensus sequences have been found in many cell types, and have been identified in the regulatory proteins of many different genes. AP-1 is largely composed of the protein products of the oncogene *c-jun*, but its binding affinity is greatly increased when it forms a complex with the protein product of the *c-fos* oncogene. The Fos and Jun proteins apparently bind to one another by hydrophobic interactions of multiple interdigitating leucine residues, a structure referred to as a "leucine zipper" (90).

Activation of gene transcription is presumably mediated by binding several other regulatory proteins to functional sequences of the IL-2 enhancer. Several protein factors bind to the IL-2 enhancer including NF-AT and Oct-1. NFAT binds to the proximal regulatory site (antigen receptor and response element-1 (ARRE-1)) between 93 and 63 base pairs upstream, and the distal regulatory site (ARRE-2) between 285 and 255 base pairs upstream of the human IL-2 enhancer. Oct-1 protein binds to NFIL-2a near the ARRE-1 (94). Based on deletion mutant analysis, the ARRE-1 site most likely plays the primary role in the function of the IL-2 enhancer (95). Also, the NFIL-2a binding site may have a negative function in resting T-cells. Therefore, this region of the IL-2 promoter may have a dual role: the suppression of the IL-2 promoter in non-activated cells and activation of its promoter after cell activation (96).

Evidence indicated that IL-2 gene activation depends on prior formation and/or activation of NF-AT, and binding of the complex to the transcriptional

enhancer site ARRE-1 and ARRE-2 (96-99). Another important regulatory site of transcription control is defined by binding of the transactivator protein TAT-1 which controls IL-2 and IL-2R gene expression (100). The present view of IL-2 gene activation is that the activation process has at least two distinct pathways, one from the antigen receptor and another by activation of PKC control IL-2 gene transcription, the two-signal model of T-cell activation.

The gene activation causes the gene transcription and the subsequent protein synthesis. These proteins are also referred to as growth factors, generally having molecular weights between 15000 and 25000 kDa (95). The expression of more than 70 molecules is sequentially regulated during growth factor synthesis. The nuclear proteins Fos, NF-AT1, and NFkB are activated immediately after cell activation, followed by interferon- γ , IL-2 between 30 and 60 min, and IL-2 receptor (IL-2R) and IL-3 at 2 h (95). The temporal order of gene activation suggests contingent regulatory events (95). Growth factors act through cellular receptors in an autocrine or synergistic fashion with other factors.

The transcription of the IL-2 receptor (IL-2R) genes is another component of the T-cell activation process which is necessary for the autocrine growth of the T-cell in response to secreted IL-2. IL-2R is expressed only at very low levels in the unstimulated lymphocyte, and up-regulation of IL-2R occurs through the binding IL-2, and activation through the TCR (101). Therefore, upon stimulation of the antigen receptor by foreign antigens the level of IL-2R

significantly increases, leading to an immune response. IL-2 induced growth of T-cells requires the formation of a trimolecular complex composed of the cytokine and two integral membrane receptor proteins which are 55 and 70 to 75 kDa in size (101). TCR:CD3 mediated stimulation of T-cells leads to increased expression of the p55 subunit of the IL-2R (101). The gene encoding the IL-2R p55 polypeptide has a 5' enhancer region that can bind PMA-inducible nuclear factors (101). The association of the two chains leads to the formation of a high affinity IL-2R that binds IL-2 and stimulates mitosis (101).

The cell cycle is traditionally divided into several distinct phases, the most dramatic of which is mitosis, the process of nuclear division, leading up to the moment of division itself. As mitosis proceeds, the cell seems to pause briefly in a state called metaphase, in which the chromosomes already duplicated are aligned on the mitotic spindle. The separation of the duplicated chromosomes marks the beginning of anaphase. During this phase the chromosomes move to the poles of the spindle where they decondense and reform intact nuclei. The cell is then pinched in two by a process called cytokinesis, which is traditionally viewed as the end of the mitotic phase (M-phase). The interval between the M phase and the beginning of DNA synthesis is called the growth phase 1 (G1-phase) and the interval between the end of DNA replication and the beginning of mitosis is called the G2-phase. G1 and G2 provide additional time for growth. The replication of DNA occurs in the synthesis phase (S-phase). G1, S, G2, and M are traditional subdivisions of the

cell cycle (102).

In S phase the synthesis of nucleotides is required in order to accommodate the increase in DNA synthesis. There are two major pathways of purine synthesis, the *de novo* and the salvage pathways. In the *de novo* pathway, the ribose phosphate portion of purine nucleotides is derived from 5'-phosphoribosyl-1-phosphate (PRPP), which is synthesised from ATP and ribose-5'-phosphate, a product of the pentose phosphate pathway. In the salvage pathway, the synthesis of nucleotides involves the reutilization of nucleotides originating from the intestinal tract or from intracellular degradation (102).

In the *de novo* pathway, the purine ring is assembled on ribose phosphate by a series of steps, the first of which is catalyzed by glutamine-PRPP amidotranferase. PRPP is also used by the purine salvage pathways, including the major pathway catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Production of an adequate level of PRPP is therefore essential for synthesis of purine ribonucleotides by either pathway. Ribonucleotides diphosphates (ADP and GDP) are converted by ribonucleotide diphosphate reductase into the corresponding deoxyribonucleotides, which are phosphorylated to produce dATP and dGTP, substrates for DNA synthesis (103).

Mitotic division of activated T-cells results in the expansion of cell clones with the same antigen specificity and thereby augments the immune response to a particular antigen. Stimulated T-cell mitotic activity can be measured

within 48 to 72 hours *in vitro*. The binding of T-cell growth factors, particularly IL-2, to their respective receptors initiates a series of incompletely understood events that culminate in the mitotic activity (101).

ii. **Mechanism of action of Cyclosporin G**

Activation of lymphocytes is a multi-step process, by inhibiting one step in the activation cascade immunosuppression would be achieved. The new immunosuppressive drugs CsG and MMF have mechanisms of action that inhibit different points in the activation process of T- cells.

The mechanism of action of CsG is thought to be identical to that of CsA. Therefore, most of the work that has been done to determine the mechanism of action of CsA is also applicable for CsG. The main *in vitro* target of CsA action is the T-lymphocyte, although other cell lineages are not completely insensitive to the drug. The effect of CsA has been evaluated in the T-lymphocyte at the level of signal recognition, signal transduction, gene activation, expression of growth factor receptors, and growth factor synthesis. Although antigen binding has not been directly studied, present experimental evidence with ligand binding to T-lymphocyte receptors does not suggest any effect of CsA at the level of ligand recognition (104). Alternative pathways of T-cell activation, e.g, mediated through binding to the CD2 or CD28 receptor associated structures, have also been examined. The recognition of CD2 or CD28 membrane proteins are not affected by CsA (95).

After signal recognition there is usually a second messenger cascade

initiated. CsA did not affect activation dependent phosphoinositol turnover in either T- or B-cells (95). Consequently, both T- and B-cells did not show any inhibition of activation-induced Ca^{2+} uptake and/or Ca^{2+} mobilization (95). Therefore, it can be assumed that PKC is still activated by phorbol ester and Ca^{2+} in the presence of CsA. The putative substrates of PKC include the τ - and δ - chains of CD3. These chains are normally phosphorylated after T-lymphocyte receptor mediated activation in the presence of CsA (105). Present data suggest that the block of CsA is distal to the Ca^{2+} and PKC signals, including increased membrane phospholipid metabolism. It has been found that CsA inhibits phospholipid turnover during lymphocyte activation and demonstrates a dose dependent inhibition of the enzyme lysolectin acyltransferase (95). However, inhibition of phospholipase A2 and phospholipid metabolism occurs only at high CsA concentrations, possibly playing a role in the toxic effects and not necessarily the immunosuppressive effects of the drug (106).

The immunosuppressive action of CsA is caused by inhibiting the signalling pathway leading to the expression of a discrete set of lymphokine genes. This has been demonstrated for the IL-2 gene, in heterogeneous T-cell populations, on cloned T-cell lines, and by *in situ* hybridization (107). The inhibition of IL-2 is crucial for the blockade of T-cell activation. Because of the central role that inhibition of IL-2 production plays in the action of CsA, focus has been placed on how CsA compounds regulate the transcriptional process.

The Handschumacher laboratory isolated an 18 kDa cytosolic protein from bovine thymus which they named cyclophilin (CyP) (107). It is a ubiquitous protein that constitutes 0.1-0.4% of total cytosolic protein (108). CyP has been shown to be the major protein responsible for the cellular uptake of CsA. CyP alone does not interact with CN (109). CyP appears to interact with CsA at amino acid (aa) 1 (mainly at C3 and C5) and with aa 2, 10, and 11 (110). The correlation between the aa recognized by CyP and the residues involved in the immunosuppressive activity (1, 2, 3, 10, 11) suggests that binding to CyP is important for CsA activity (110). Unbound CyP possesses enzymatic activity and has been identified as a peptidyl-prolyl cis-trans isomerase (PPIase). CsA binding to CyP inhibits the PPIase activity (111). However, both genetic and pharmacologic experiments rule out the possibility that the PPIase activity interferes with signal transduction (107). The PPIase activity is a fortuitous coincidence; the evidence presented thus far linking CyP to the critical site of action of CsA is largely circumstantial (107). Both genetic and pharmacologic studies show that the drug-protein complex plays an active role in the inhibition of a signalling pathway. It has been proposed that both CNA and CNB contribute to the binding of CsA-CyP complexes and that the binding site resides at, or very near, the interface between the two subunits (109).

Two regions in the IL-2 gene appear to be particularly drug-sensitive: the ARRE-1 and ARRE-2 site which binds NF-AT, a factor that is absent in unstimulated T-cells but appears 10-25 min after activation; and the NFIL-2a

site, near the ARRE-1 site, which binds the constitutively expressed factor Oct-1 (107). The ability of CsA to block the transcriptional activity mediated by NF-AT and Oct-1 is due to interference with the transport of these factors into the nucleus. The CsA-CyP complex binds and inhibits the activation of CN, by causing a conformational change between CNA and CNB (109). This complex of CsA-CyP-CN prevents the dephosphorylation of the NF-ATc, thus preventing it from crossing the membrane and initiating the transcription of IL-2 genes.

CsA inhibits not only the transcription of IL-2 but a variety of cytokines and protooncogenes. Because of the central regulatory role of ARRE-1, the effect of CsA was evaluated on this DNA binding site. Gene constructs containing this sequence are suppressible by CsA (94). Therefore, the initiation of DNA transcription is inhibited. Depending on the mode of the signal pathway (either through TCR stimulation, or through the Ca^{2+} and/or PKC signal), activation of the genetic program and sensitivity to CsA may differ (95). For example, anti-CD3-induced lymphocyte proliferation is highly sensitive to the inhibitory action of CsA, but activation of PKC by phorbol esters is CsA resistant; activation by calcium ionophores is highly sensitive to the inhibitory effect of CsA (105).

The effect of CsA on the IL-2 receptor has been extensively investigated. No effect was found on receptor expression. An inhibitory effect of CsA on mitogen-induced IL-2 synthesis in murine and human lymphocytes and cell lines

is well established (99,111). Whereas the synthesis of IL-1 α , IL-1 β and granulocyte macrophage colony-stimulating factor (GM-CSF) are CsA resistant, the synthesis of IL-3, IL-4, and IL-6 and interferon- γ synthesis are inhibited by CsA. Effects on other hematopoietic growth and differentiation factors such as granulocyte colony stimulating factor (G-CSF), macrophage/monocyte colony stimulating factor (M-CSF), IL-7 and IL-8, have not yet been reported (95). CsA inhibits the synthesis of a broad spectrum of cytokines after cell activation.

The inhibition of gene activation is thought to be the main pathway for the effect seen with CsA. If gene activation does not occur, subsequent mRNA transcription and protein synthesis will also be inhibited. Therefore, no growth factors or receptors, such as IL-2 and IL-2 receptor, will be synthesized.

iii. Mechanism of action of mycophenolic acid

Whereas CsA inhibits early events in the activation of lymphocytes, MPA inhibits the activation at a later stage by preventing the synthesis of DNA, by inhibition of inosine monophosphate dehydrogenase (IMPDH, E1.2.1.14), and arresting the cells in the G1 phase of the cell cycle. Therefore, MPA has the potential to act synergistically with CsA.

Inosine monophosphate (IMP) is at the branch point in purine nucleotide synthesis, since it can be converted to adenosine monophosphate (AMP) or guanosine monophosphate (GMP). Two enzymes decrease levels of adenosine nucleotides relative to guanosine namely, adenosine deaminase (ADA) and IMPDH (103). Therefore, an effective therapeutic strategy would be to target

these enzymes. MPA is a potent inhibitor of human IMPDH, and is a potentially important chemotherapeutic agent given its branch point in purine biosynthesis. Concentrations of MPA that are clinically attainable readily inhibit the proliferative response of human peripheral blood mononuclear cells to phytohemagglutinin (PHA), (T-cell mitogen), pokeweed mitogen (T-dependent B-cell mitogen), and Staphylococcus protein A sepharose (B-cell mitogen) (112). *In vitro* the IC_{50} for MPA is less than 100 nM on mixed lymphocyte cultures. At this concentration the drug has no antiproliferative effects on fibroblasts, endothelial cells, or on any other cell type studied. Thus the drug has greater *in vitro* effects on B- and T-cells than on any other cell type. MPA also inhibits proliferation of B-cells and thus, *in vitro*, prevents primary polyclonal and antigen-specific secondary antibody responses to antigens (113).

The action of MPA on IMPDH activity in cells will ultimately lead to the cellular depletion of GMP, and similarly GTP. Guanosine phosphates are required for the synthesis of DNA necessary for cell growth and replication. Consequently, MPA has its greatest effect during the S-phase of the cell cycle (114,115). GTP is also required for specific cellular reactions, such as initiation of polypeptide chain synthesis and elongation (116). GTP also takes part in membrane signal transduction pathways, examples of which involve β_2 -adrenergic, norepinephrine, and dopamine receptors (116). Alterations in the cellular concentration of the guanosine phosphates will have a profound effect on lymphocyte functioning. Depletion of GTP pools may lead to the inhibition

of T-cell activation by decreasing the signal transduction via the TCR/CD3 complex. Signal transduction through the T-cell receptor is thought to be mediated by G-protein coupling of the receptor to phospholipase C (117). Depletion of GTP also inhibits the transfer of fucose and mannose residues to glycoproteins, some of which are adhesion molecules facilitating the attachment of leucocytes to endothelial cells and to target cells during rejection (117).

The depletion of the guanosine nucleotides may also adversely affect the concentration of adenosine nucleotides. The adenosine nucleotide pool is both abundant and important for cellular survival. Adenosine triphosphate (ATP) is required as an energy source for many cellular functions, including protein synthesis, carbohydrate metabolism, and DNA replication (116). Consequently, alterations in this pool could also have a significant effect on cellular function.

IMPDH is considered a key enzyme in the *de novo* biosynthesis of purine nucleotides, in particular guanosine monophosphate (GMP). Allison et al. report both human T- and B-lymphocytes are sensitive to MPA, whereas other cell lines are not (118,113). The relatively high degree of cellular selectivity of MPA is based on the observation that lymphocytes are more dependent on the *de novo* pathways of purine synthesis than are other cell types. However, dependence on *de novo* purine synthesis has been observed for most proliferating cells (113,65). Not only *de novo* enzymes but enzymes of the salvage pathways, for example the purine phosphoribosyl transferases, are

elevated during proliferation (65). In lymphocytes, *de novo* purine synthesis is not stringently segregated from salvage activities in lymphocytes. Activated lymphocytes may simply require a large flux through all purine biosynthetic pathways. Any mechanism that attenuates this flux may compromise immune function. The singular advantage of selective inhibition of guanine nucleotide synthesis may be the tolerance of other cells to modest decreases in guanine synthetic pathways, not their relative reliance on the *de novo* versus salvage synthesis (65).

The IMPDH gene expression is regulated inversely by the intracellular level of guanine ribonucleotides (119). Treatment with exogenous guanosine increased the level of intracellular guanine ribonucleotides and subsequently reduced IMPDH steady state mRNA levels in a time- and dose-dependent manner (119). Conversely, MPA treatment diminished the level of guanine ribonucleotides and increased the IMPDH mRNA levels (120). The effect on IMPDH mRNA levels could be negated by co-treatment with guanosine and MPA (119). The regulation of IMPDH gene expression was not due to changes in transcription and elongation of mRNA, or changes in the stability in the cytoplasm, but rather to alterations in the levels of IMPDH mRNA in the nucleus (119). Therefore, IMPDH gene expression is regulated by post-translational, nuclear events in response to fluctuation in the intracellular guanine ribonucleotides (119).

PRPP-synthase in human lymphocytes is inhibited by adenosine

nucleotides and activated by guanine nucleotides (119). Such regulation is a critical factor in the therapeutic strategy of MPA. An excess of adenosine nucleotides and/or a decrease of guanosine nucleotides can decrease the pool of PRPP, and an excess of dATP and/or a decrease in dGTP can inhibit ribonucleotides reductase activity. Therefore, adequate levels of guanosine and deoxyguanosine are required for proliferative responses of lymphocytes to antigenic and mitogenic stimulation.

Because of the importance of guanosine and deoxyguanosine nucleotides in activation of PRPP synthase and ribonucleotide reductase, depletion of GMP (and consequently GTP and dGTP) by inhibiting IMPDH would have an antiproliferative effect. Furthermore, since lymphocytes rely heavily on the *de novo* purine synthesis pathways, antiproliferative effects produced this way would be more potent on lymphocytes than any other cell types (103).

IMPDH catalyses the irreversible NAD-dependent oxidation at C2 of IMP (41). The reaction follows ordered bi-bi kinetics (121). IMP is the first substrate to be associated with the enzyme, followed by the association of NAD to form a tertiary complex. After hydride transfer NADH is the first product to dissociate, and XMP is the last, liberating free enzyme to complete the catalytic cycle. The stereochemistry of hydride transfer at the C4 of the NAD nicotinamide has been determined to be B-side of pro-S (121). Evidence suggests that the rate limiting step does not involve binding of NAD to the enzyme (122).

The three dimensional structure of IMPDH is not known. The bi-bi substrate reaction implies two active-site pockets, one each for the substrate pairs IMP/XMP and NAD/NADH. The steps in the reaction pathway that lead to an intermediate that is capable of transferring a hydride atom from the C2 position of IMP to NAD has not been elucidated. Two mechanisms have been proposed for this reaction. One consists of the general base-assisted addition of hydroxide at the C2 position to form a tetrahedral centre, followed by hydride transfer. The second mechanism involves attack by a nucleophile residue of the protein at this carbon to form a tetrahedral intermediate. Upon hydride transfer, this intermediate collapses to form a covalent enzyme substrate complex. Hydrolysis of the covalent intermediate form frees the enzyme for another catalytic cycle after dissociation (123,124).

The consensus nucleotide-binding motif of β - α - β had been predicted in *Escherichia coli* IMPDH on the basis of steric and physicochemical properties of amino acids from pattern searches of protein sequence databases (125). The domains are located from Asp-319-Lys-349 in human IMPDHs, and the sequences are well conserved in IMPDHs from several different species (126-128). An active site cysteine has been postulated for the nucleotide-binding domain of IMPDH, based on the identification of a single conserved cysteine (Cys) (sulfhydryl group) in the sequences of IMPDH and closely related GMP reductase from numerous organisms (129). Identification of a conserved Cys³³¹ near the IMP-binding site in both type I and II proteins suggests it has a

possible role in covalent catalysis but does not, however, exclude a noncovalent catalytic mechanism in which Cys³³¹ may function as a general acid or base. Finally, it has been suggested that the IMP binding site includes a thiol group (130,74).

IMPDH is inhibited by a number of ribonucleotides, including XMP, GMP, AMP, and NADH. Inhibition of the enzymes by XMP and GMP suggests feedback regulation by proximal and distal products of the GMP biosynthetic pathway. Metabolic control regulation of intracellular IMPDH activity is likely to operate via substrate or product inhibition kinetics. However, allosteric properties have not been observed for the human enzymes (122).

The IMPDH isolated from Bacillus subtilis is largely a membrane bound enzyme at an early phase of active protein synthesis, and then becomes progressively solubilized. The soluble and membrane bound forms of IMPDH have three notable differences. First, the soluble enzyme has about twice the specific activity of the membrane bound form. Second, the soluble species is unstable when stored, but can be stabilized in the presence of membrane material. Finally, the solubilized IMPDH has a higher tendency to exhibit multiple molecular forms on gel filtration. IMPDH is composed of readily dissociable tetramers (41,122,131,132).

Human IMPDH exists in two isoforms. In lymphocytes type I is constitutive, and type II is induced upon activation (41). Recent studies have confirmed that both are tetramers composed of four 56 kDa monomers with

similar K_i values for XMP and NADH (122). Both enzymes have ordered bi-bi kinetic mechanisms (122). This model indicates that both substrates bind to the enzyme before either product is released. It also indicates that the substrates bind in an obligate order IMP preceding NAD, and that the products disassociate in an obligate order as well, NADH preceding XMP. The same underlying mechanism of inhibition is demonstrated for both human type I and type II (122). Both type I and type II exhibit similar affinities for both substrates, IMP and NAD (122).

IMPDH enzymatic activity is encoded by two distinct genes termed IMPDH I and IMPDH II (133,134). Northern Blot analysis using cDNA probes for type I and type II IMPDH's demonstrated that type I mRNA was the dominant species in normal leucocytes and lymphocytes. The normally low levels of type II IMPDH mRNA were up-regulated markedly by phytohemagglutinin stimulation or by Epstein-Barr viral transformation (135). Conversely, the enhanced levels of type II mRNA, protein, and total IMPDH activity in leukemic and melanoma cells, were down regulated during differentiation induced by retinoic acid, 12-o-tetradecanoylphorbol-13-acetate or dimethylsulfoxide (120,123,135-138). The proteins encoded by the cDNAs from these IMPDH type I and II genes contains 514 amino acid (aa) with 84% sequence identity (133). 84 out of the 514 aa are substituted. Out of those 84 aa changes, 52 are conservative and 32 diverge with respect to their chemical properties (133). The chromosomal localization and structure of the

gene encoding IMPDH type II was found to be 3p21.2 to p24.2 (139). Two overlapping yeast artificial chromosome clones containing the full gene for type II IMPDH were isolated and a physical map of 117 kb of human genomic DNA in this region of chromosome 3 was constructed (139). The gene for type II IMPDH was localized, oriented on this map, and found to span no more than 12.5 kb (139).

Type II IMPDH expression declines in cells induced to undergo terminal differentiation (135). Modulation of IMPDH expression in tissues and cells appears to be controlled by synthesis of the type II isoform. Specific inhibition of the type II isoform has been proposed as a selective method for achieving antiproliferative and immunosuppressive effects (137,125). MPA is a specific inhibitor of IMPDH from many different species (140). Even small changes in the structure of MPA (e.g. methylation, reduction of the double bond, etc.) eliminates drug activity (140). MPA displayed inhibition constants in the order of 10^{-4} - 10^{-9} M against both isoforms of IMPDH, which distinguishes it as a tight binding inhibitor (122). MPA is a noncompetitive inhibitor for both IMP and NAD. This is consistent with a mechanism of inhibition in which MPA binds to the enzyme after association of all substrates, possibly after the hydride transfer reaction. It has been suggested that MPA binds to the enzyme after a tertiary complex is formed of enzyme, IMP and NAD^+ (122,41). MPA binds to a single site on IMPDH, possibly close to the dinucleotide site (140).

The type II isoform exhibits a 4 fold increased sensitivity to MPA

compared to type I isoform (122,41). However, the greater inhibition of IMPDH type II may explain, in part, the relatively mild side effects of MMF observed (76). The higher sensitivity of type II IMPDH to MPA may represent an exploitable difference between the isoforms since type II IMPDH is up-regulated in activated lymphocytes. Substrate accumulation in the form of IMP is a documented consequence of IMPDH inhibition. Substrate concentrations increase due to blockage of their metabolism by an inhibited enzyme. The IMP intracellular concentration is increased 10-fold or more when cells are incubated with IMPDH inhibitors such as MPA (141). The noncompetitive kinetics of inhibition of MPA against human IMPDH means that inhibition is augmented by the binding of substrates to the enzyme, not diminished as is the case for competitive inhibitors. This is an advantage of noncompetitive inhibitors over competitive inhibitors, such as AZA. A competitive inhibitor is much less effective when substrate concentrations increase, whereas noncompetitive inhibition cannot be overcome by the elevation of substrate concentrations. A noncompetitive inhibitor is of particular benefit as a drug since substrate accumulation potentiates inhibition. MPA binds to the dinucleotide site of IMPDH (140). The binding of MPA to the NADH binding site is particularly interesting because there are many compounds (Cibacron Blue, salicylate, anilino-naphthalenesulfonates, tetraiodofluorescein) that bind to the adenosine portion of NAD binding sites. These results suggest that more potent MPA analogues might be designed by adding a moiety that would bind in the

adenosine sub-site (140). For the T_{foetus} enzyme, multiple inhibitor experiments with NAD analogues and MPA suggest that MPA binds to a portion of the NAD binding pocket of the enzyme (140,142). The development of type-II specific drugs may, therefore, provide improved selectivity in immunosuppressive therapy.

5. PHARMACOKINETICS AND METABOLISM

i. Cyclosporin G

Pharmacokinetic studies in animals have reported CsG to have a higher and lower clearance rate as compared to CsA, depending on the animal model studied (143,144). The conflicting findings may have been caused, in part, by differences in the crystal form of CsG or the vehicle used to administer the drug in these studies (143,144). In normal volunteers administered a single dose of CsG and CsA in a crossover fashion, similar blood concentrations and half lives of 8 h with both drugs were attained. The average elimination half life of 18 h for CsG in patients with end-stage renal failure is similar to that generally reported for CsA in renal transplant patients (145). However, in a separate study, the blood concentrations of CsG in renal transplant patients were significantly higher compared to CsA after 4 weeks of drug treatment even though doses were similar (60).

CsG, like CsA, is extensively metabolized by the liver in both humans and animals by the cytochrome P-450 system (57,58,146-152). Two

isoenzymes have been shown to be responsible for the metabolism of CsA. First, the P-450 IIIa (P-450 NF) is responsible for the metabolism of many hydrophobic compounds including nifedipine, cortisol, erythromycin, and quinidine (149-151). A second cytochrome P-450, hpCN3, has also been found to be involved in CsA metabolism (152).

CsG and its metabolites are eliminated through the bile although a small but significant amount is excreted in the urine (147,148). Seven major CsG metabolites have been isolated from the bile or urine from a number of animal species as well as man using a number of different HPLC procedures (56). The metabolites formed are primarily demethylated and mono- and dihydroxylated analogues of CsG as shown in Table I-1. The nomenclature of these metabolites is based on the structural modifications. The metabolic pathways for CsG and resulting metabolite structures are similar to those previously reported for CsA (56-58). In all metabolites identified to date, the cyclic structure has been preserved. The reactions involved in the biotransformation are linked to N-demethylation, hydroxylation, oxidation, and cyclization (57,58,146,129,153-157). N-demethylation appears to occur only at the N-methyllucine residue found at aa 4. Hydroxylation is restricted to the alkyl-position of aa 1 and the γ -position of N-methyllucine at aa 4, 6, and 9. Intramolecular ether formation has been observed at aa 1 (furan ring formation). The proposed biotransformation pathways of CsG suggest that metabolites GM1, GM9, GM4N should be considered as primary metabolites, with the

Table I-1 Structural Modifications of CsG Metabolites

CsG metabolite	Modification
GM1	hydroxylation aa 1*
GM9	hydroxylation aa 9
GM4N	demethylation aa 4
GM1c	hydroxylation, reduction aa 1
GM1c9	hydroxlation aa 9
GM19	hydroxylation aa 1 & 9

amino acid (aa)

* amino acid 1

secondary metabolites GM19, GM1c, GM1c9, and GM4N9 arising from the primary metabolites (158,147,148).

Numerous drugs interfere with the metabolism of CsA (159). The concurrent use of such drugs can significantly alter the PK of CsA (160,159). Drugs that induce cytochrome P-450, such as rifampin, phenytoin, phenobarbital, and carbamazepine, can accelerate metabolism and reduce CsA in blood levels. Erythromycin, on the other hand, competes with CsA for binding sites on the enzyme by binding to the P-450 complex, forming an inactive complex, leading to increased levels of CsA (159,161). Ketoconazole leads to increased blood levels by reducing P-450 activity (162).

The therapeutic monitoring of CsG has been included as an integral part of the clinical trials with the drug (63,64). On a dose for dose basis the blood concentrations of CsG tend to be higher than those for CsA (63,64). Many of the centres involved in the clinical trials have used commercial immunoassays for the measurement of CsG (63,64). These assays were originally developed for the measurement of CsA and subsequently adapted for the measurement of CsG due to the significant cross-reactivity of the latter drug in those assays (63,64).

Measurement of CsA can be achieved using a variety of different analytical methods. Validated high-performance liquid chromatographic (HPLC) methods that measure the parent drug specifically can be used as reference procedures. A definitive reference method according to the International

Society of Clinical Chemists (IFCC) criteria is not yet available (163). Recent reports have indicated the increasing use of semi-automated immunoassay techniques involving selective monoclonal antibodies rather than HPLC for routine use, with a predominance of the INCstar CYCLO-Trac SP RIA and Abbott TDx monoclonal antibody FPIA (164). Concentration of CsA metabolites in renal transplant recipients has been previously established. In blood the primary metabolites AM1 and AM9 were present in ratios compared to parent drug of 1.5:1 and 0.75:1 respectively. The secondary metabolites AM19 (0.69), AM1c (0.40), and AM1c9 (0.05) are in lower concentrations than is parent CsA (165-169). Of all available methods used in clinical and research studies, the FPIA has demonstrated the best intra- and inter-lab reproducibility (170). Although the methods most commonly in use have a high specificity for the parent compound, it is well established that they do not give identical results. The difference between the values obtained using HPLC and the high specificity immunoassays is, in part, due to the cross-reactivity towards the CsA metabolites (171).

Throughout the clinical trials with CsG, it was assumed that therapeutic drug monitoring protocols developed for CsA could be adapted for use with CsG. Based on differences between the two drugs, it would be expected that difficulties would occur with such an approach. CsG should be treated as a new drug with the development of independent therapeutic monitoring guidelines. CsG has a temperature- and concentration- dependent distribution

between erythrocytes and plasma (55). Subsequent to incubation for 30 min at 37°C: the plasma whole blood ratio was relatively constant at 0.7-0.8 up to a CsG concentration of 1000 $\mu\text{g/L}$. At 5000 $\mu\text{g/L}$, this increased to 1.0-1.1 (55). These ratios are higher than those previously reported for CsA (0.5-0.6) (172). This indicates that a larger proportion of CsG is present in the plasma fraction as compared to CsA. The binding of CsG to granulocytes (25%) and lymphocytes (6%), as well as the free fraction of the drug in plasma (13-17%), was similar to that previously reported for CsA (55,172). As is the case for CsA in plasma, CsG is primarily associated with high density lipoprotein (50-60%) and to a lesser degree with low density (20-30%) and very low density (10%) lipoproteins (55).

The primary metabolites, GM1 and GM9, are sequestered to a greater extent within erythrocytes as compared to the parent drug, which is consistent with that reported with the comparable CsA metabolites (56,57). In 1° and 2° mixed lymphocyte reactions, the immunosuppressive activities of CsG metabolites are similar to those of CsA metabolites, being less than 10% of parent drug (56,57). However, little was known about the steady state concentrations of CsG metabolites, the role they have in the side-effects of the drug and the impact they may have on the overall monitoring of the drug by immunoassays since the concentration of the metabolites is not known.

The clinical relevance of CsG metabolites is unknown and the relevance of CsA metabolites is still controversial. Some immunosuppressive activity has

been found *in vitro* for CsA metabolites AM1, AM9, and AM4n (164). Although over immunosuppression induced by high concentrations of CsA metabolites could increase the risks of serious infection, the relative toxicities of the metabolites are probably more important clinically (164). Studies involving CsA metabolites indicated that AM4n and AM9 were the most cytotoxic metabolites examined, having potency ratios of 0.28-0.56 with respect to parent CsA for the inhibition of cell growth and DNA synthesis. However, some of the CsA metabolites were equipotent as parent drug in decreasing prostacyclin production. AM19 and AM1c resulted in increased endothelin production, whereas CsA resulted in a slight decrease (173). This suggests that CsA metabolites may contribute to the pathogenesis of CsA induced renal side-effects. CsG has been shown to induce fewer renal side-effect than CsA, however, little is known regarding whether this is due to the differential effects of metabolites between the two drugs.

ii. **Mycophenolate mofetil**

Mycophenolic acid (MPA) has a low bioavailability in humans (71) and in the search for selective nontoxic analogues with a greater bioavailability many synthetic and semi-synthetic variants were evaluated. Hydrogenation of the side-chain double bond was known to cause marked loss of antimitotic potency. The π -electron character in the appropriate positions on the side chain groups relative to the nucleus and the terminal carboxyl is required (174). MMF (2-(4-morpholino)ethyl-(E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-

5-isobenzofuranyl)-4-methyl-4-hexanoate) is the morpholinoethyl ester of MPA. In primates MMF has a two fold greater bioavailability than MPA (66). Once absorbed, MMF is rapidly hydrolysed to MPA by plasma, tissue, and cytosolic esterases to yield the active compound, MPA (175,66,71). The immunosuppressive activity of MMF is expressed only after hydrolysis, since the terminal carboxyl group is necessary for activity (66,72).

Significant variations in the pharmacokinetics of MMF were found in renal transplant patients. The concentration maximum (C_{max}) and area under the curve (AUC) values are significantly lower in the early post-transplant period (within the first 40 days) compared to times > than 3 months (176-178). A recent consensus report suggested that altered absorption in the early post-transplant period does not account for the observed change because the MPA AUC values at this point for oral MMF are comparable to the AUC values obtained in the same renal transplant patients after an equivalent dose of MMF administered by the IV route (176,177). Thus the lower values for C_{max} and AUC₀₋₁₂ observed in the early post-transplant period might be due to absorption, distribution, or metabolism, or a combination of these. Other possible explanations could be decreased plasma protein binding or the involvement of enterohepatic circulation. Decreased plasma protein binding is associated with the early post-transplant period and may lead to an increased volume of distribution for MPA, and an increased clearance by the liver of free drug. This will lower the total MPA concentrations. The enterohepatic recirculation

process increases with time post-transplant, therefore, there is a slow accumulation of MPA and MPAG, or both (176,177).

Drugs that displace another drug may transiently increase free concentration. Several drugs have been evaluated for their effect on the binding of MPA (179). Many commonly used immunosuppressive drugs including; CsA, FK-506, and prednisone have no effect on MPA binding. Other commonly prescribed drugs, such as: coumadin, digoxin, phenytoin, ranitidine, cimetidine, heparin, acetaminophen, and ibuprofen have no effect on the binding of MPA. However, sodium salicylate, furosemide, and ethanol decrease the binding of MPA (179).

MPA is rapidly inactivated by glucuronyltransferase and β -glucuronidases to form mycophenolic acid glucuronide (MPAG). Glucuronidation, at the hydroxyl group of MPA is thought to account for its relatively low *in vitro* potency (180,184). MPAG accounts for 80-90% of the total circulating drug *in vivo* (68,66,185,186). MPAG undergoes deglucuronidation and reabsorption as MPA during the process of entero-hepatic circulation (122,176). This mode of metabolism decreases the fraction of free MPA in circulation, but appears to extend the effective half-life (177). Experimental evidence for this includes the appearance of secondary peaks in plasma MPA concentration time profiles at 6 to 12 h and again, although of lesser magnitude, at 24 h; the presence of MPAG in the bile of liver transplant patients; and the observation that cholestyramine treatment reduced, the average plasma AUC. The initial

absorption, rate, and peak concentration characteristics for MPA were not affected, but the remainder of the MPA concentration-time curve was significantly reduced, which is consistent with cholestyramine binding to MPA and MPAG in the GI tract (177).

II. OBJECTIVES AND RATIONAL

The therapeutic monitoring of CsG and MMF have been recommended to optimize immunosuppression while minimizing toxicity for both drugs (63,64,177). Therapeutic monitoring has been an integral part of the clinical investigations with CsG. Throughout the clinical trials with CsG, it was generally assumed that therapeutic monitoring protocols developed for CsA could be adapted for use with CsG. CsA metabolites have a clinical significance index that is low compared to the parent drug, thus assays specific for CsA have been adapted for routine TDM (57). In monitoring of CsG the assumption was made that the role of metabolites was the same as for CsA. However, this had not been determined, therefore, it is not necessarily appropriate to assume that monitoring strategies acceptable for CsA are applicable for CsG. The blood distribution and the PK of CsG have been previously determined and shown to be comparable to CsA (55,143,144). However, the role of CsG metabolites in overall immunosuppression and toxicity are not known. If contributions by metabolites as assessed by the clinical significance index, are sufficiently low it is then hypothesized that CsG should be monitored in a manner similar to CsA, and assays specific for parent drug using whole blood for analysis should be used. In order to achieve this end, the objectives of this study are the following:

- 1) To assess the degree of cross-reactivity of CsG metabolites in CsA immunoassays.
- 2) To determine the steady state concentration of CsG metabolites in renal transplant patients
- 3) To determine the in vitro toxicity of CsG metabolites on primary renal cell lines.

Less is known regarding the optimal monitoring protocols for MMF. It is presently not known whether therapeutic monitoring of MMF will be necessary to minimize toxicity or optimize immunosuppressive efficacy. MMF has been used in clinical trials to prevent graft rejection and in the treatment of ongoing rejection. All studies to date were performed using a fixed dose of drug (78,76). Although MMF has been shown to be effective in these studies, since monitoring was not performed it is not possible to determine if TDM would improve patient outcome. The most appropriate medium for the analysis of MMF is not known since the distribution of the drug in whole blood has not been determined. Consequently, it is not known whether the measurement of the total MPA concentration alone, the free fraction of the drug, or if measurement of total drug plus the metabolite, MPAG, should be monitored. Such information would be useful to establish guidelines for monitoring of the

drug to facilitate consistency in therapeutic evaluations. It has been suggested that traditional therapeutic monitoring may not accurately reflect the status of the patients and that PD monitoring (measurement of the biological response) would be more appropriate for assessment of the immunological or toxicological state of the patient (179). The above studies should provide information on whether a pharmacodynamic approach provides a better indication of immunosuppression or toxicity. It is hypothesized that PD monitoring would allow for dosage adjustments to optimize immunosuppression and minimize drug induced side-effects. In order to achieve this end, the objectives of this study are as follows:

- 1) To determine the distribution of MPA in blood
- 2) To establish an assay for the monitoring of IMPDH activity in whole blood.
- 3) To determine the relationship of IMPDH activity and MPA concentrations in animal models
- 4) To evaluate the use of pharmacodynamic monitoring in rabbit heterotopic heart transplant model.

- 5) To evaluate the relationship of MPA levels and IMPDH activity in a renal transplant recipients.

III. CROSS REACTIVITY OF CYCLOSPORIN G (CsG) METABOLITES IN CYCLOSPORIN A (CsA) IMMUNOASSAYS

1. RATIONALE:

Many of the centres involved in the clinical trials with CsG used commercial immunoassays for the measurement of the drug (66,64). These assays were originally developed for the measurement of CsA and subsequently adapted for the measurement of CsG due to the significant cross-reactivity of the latter drug in these assays (63,64,187). However, the cross-reactivities of CsG metabolites in these assays is not known. The cross-reactivity of CsG metabolites in a number of commercial assays used for the measurement of CsA was investigated.

2. MATERIAL AND METHODS

a. Source of drugs and specimens

CsG and CsA were obtained as gifts from Sandoz Inc (East Hanover NJ). CsG metabolites were isolated from urine obtained from subjects receiving the drug. To 40-50 mL of urine (pH adjusted to 10.0 with 2 mol/L NaOH) 50-60 ml of HPLC grade diethyl ether were added. The mixture was shaken for 10 min, and the urine layer removed after centrifugation. Using the same ether layer, two or three more such portions of urine were extracted. To the ether layer 50 mL of 0.2 mol/L HCL were added and the mixture was shaken for 10

min. After centrifugation the ether layer was removed and evaporated to dryness under a stream of air. The residue was reconstituted in 500 μ L of mobile phase (acetonitrile/methanol/water: 40/20/40 by volume). Subsequent to addition of 500 μ L of hexane, the sample was vortex mixed for 30 s, then centrifuged (2000 $\times$ g for 5 min). The hexane (upper) layer was discarded and the remaining solvent layer was collected for subsequent separation by HPLC.

Isocratic chromatographic separation of metabolites was achieved using a 25x0.46 cm 5- μ m Spherasorb C-8 HPLC column (Chromatography Sciences Inc., Rexdale, Ontario, Canada) preceded by a precolumn of pellicular silica. The mobile phase used is described above. In some instances a decreased concentration of acetonitrile in the mobile phase was used to improve chromatographic resolution. The flow rate was 1.0 mL/min; the column temperature was maintained at 70°C. Eluent fractions corresponding to the peaks were collected manually, and the mobile phase was evaporated under low heat (50°C). Corresponding fractions from multiple injections were pooled for subsequent experiments.

The structure and purity of the metabolites tested (GM1, GM9, GM1c, GM19, GM4N, GM1c9) were confirmed by FAB/MS and ^{13} C NMR at the University of Manitoba (56). All metabolites tested had a purity of > 97%. Whole blood was obtained from the Red Cross, with ACD used as an anticoagulant, and from a number of laboratory staff using EDTA as anticoagulant.

b. RIA

CsG and metabolites were quantified by two different RIA's as follows; Sandimmune RIA (Sandoz Inc. Basle CH), which includes a monoclonal antibody that was selective (SS) and one that was non-selective (NS) for parent drug, and an alternate selective RIA (Cyclotrac-SP, Incstar Inc. Stillwater, MN) (CT). All assays were performed as previously described (188,189). For certain experiments with both RIAs, the CsA calibrators supplied by the manufacturer were replaced with whole blood CsG calibrators prepared in our laboratory. Calibrators were routinely prepared with blood containing ACD as anticoagulant. In some instances, where indicated, they were also prepared with blood containing EDTA as anticoagulant. The concentrations of CsG in these calibrators as confirmed by HPLC were 0, 25, 50, 100, 200, 400, and 800 $\mu\text{g/L}$.

c. Fluorescence polarization and enzyme immunoassays

CsG and metabolites were quantified by fluorescent polarization immunoassay (FPIA) using reagents provided in kit form (Abbott Diagnostics Abbott Park IL) (55). Parent drug was also measured by Enzyme Multiplied Immunoassay (EMIT) (Syva Co. Palo Alto CA) on a Cobas Mira-S analyzer (Hoffmann La Roche Ltd. Missassauga, ON) (190). Both assays were calibrated with CsA standards as supplied by the manufacturer. For certain experiments both assays were calibrated with whole blood CsG calibrators prepared in our laboratory having the same concentration as the respective CsA

calibrators (0, 100, 250, 500, 1000, 1500 $\mu\text{g/L}$).

d. Cross-reactivity determinations

Aliquots of either drug free whole blood or blood containing CsG at a concentration of 200 $\mu\text{g/L}$, were individually supplemented with the CsG metabolites. All assays were performed in duplicate on at least 3 separate days ($n=6$). We determined the cross-reactivity of CsG metabolites in assays calibrated with CsG using the following equation: (metabolite measured as CsG/metabolite added) $\times$ 100. For assays calibrated with CsA the following equation was used: (metabolite as measured as CsA/metabolite added) $\times$ (100/cross-reactivity of CsG) $\times$ 100.

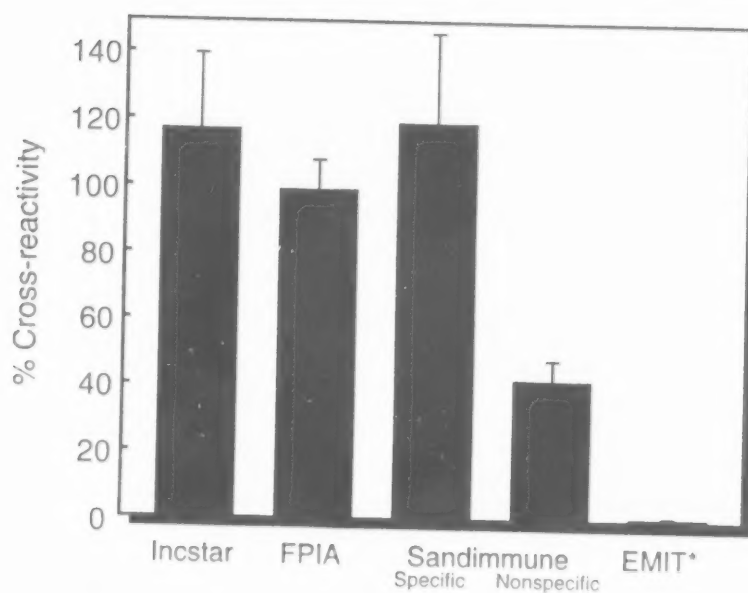
3. RESULTS

a. Cross-reactivity of CsG

The mean cross-reactivity of CsG in commercially available immunoassays at a concentration of 250 $\mu\text{g/L}$ in the various assays tested is shown in Figure III-1. The exception was the EMIT assay where a concentration of 5000 $\mu\text{g/L}$ was used. The cross-reactivity of CsA did not significantly vary over the concentration range investigated (50-1000 $\mu\text{g/L}$). CsG exhibited a cross-reactivity of approximately 100% in the FPIA and 117% in the SS and CT assays. In contrast, 40% and 2% cross-reactivity were seen with the NS and EMIT assays, respectively. The values established for the cross-reactivity of CsG in assays calibrated with CsA were used to correct for

FIGURE III-1

Cross-reactivity of Cyclosporin G (CsG) in Cyclosporin A (CsA) Immunoassays



Cross-reactivity of CsG in CsA Immunoassays. Values were calculated at a concentration of 250 $\mu\text{g/L}$ with the exception of the EMIT assay where a concentration of 5000 $\mu\text{g/L}$ was used. Results expressed as $\bar{x} \pm \text{SD}$ ($n=6$).

CsG metabolite cross-reactivities in such assays as described in the Methods Section. Due to the lack of cross-reactivity of CsG in the EMIT assay, further investigation of this assay with CsG metabolites was not performed.

b. Cross-reactivity of CsG metabolites

The cross-reactivities of CsG metabolites in assays calibrated with CsA and CsG are shown in Tables III-1 and III-2, respectively. A comparison of the cross-reactivities among the assays at metabolite concentrations of 5000 $\mu\text{g/L}$ (SS, FPIA, and CT) and 500 $\mu\text{g/L}$ (NS) is shown in Figures III-2 and III-3. The lower concentration was used for the NS assay since higher concentrations of metabolites resulted in values that were beyond the linear range of the assay (0-800 $\mu\text{g/L}$). The cross-reactivities did not exhibit a significant change over the concentration range tested (500 - 5000 $\mu\text{g/L}$). The NS assay exhibited the most cross-reactivity with metabolites. With this assay GM1c exhibited a cross-reactivity of greater than $128 \pm 9.6\%$ in assays calibrated with CsA. Substantial cross-reactivities were also found for GM1, GM9, GM1c9 and GM19 with this assay. In contrast, in the other assays (SS, FPIA, CT), the cross-reactivities of all metabolites was $< 12\%$. The most cross-reactivity was found with metabolites having single modifications (primary metabolites) GM9, GM4N and GM1. The secondary metabolites (GM1c9, GM19) having more than one modification exhibited substantially less cross-reactivity. The exception to this was GM1c, a secondary metabolite which exhibits a similar cross-reactivity as the primary metabolites in the SS, FPIA and CT assays. The

Table III-1

Cross-reactivity of Cyclosporin G (CsG) metabolites in assays calibrated with Cyclosporin A (CsA)¹

Metabolite	Concentration ($\mu\text{g/L}$)	Sandimmune Specific		Sandimmune Non-specific		FPIA		Cyclotrac-SP	
		mean %	SD	mean %	SD	mean %	SD	mean %	SD
GM1 ²	5000	2.0	0.9	>HS ⁴		8.0	0.6	1.0	0.3
	2500	1.3	0.2	44.5	10.2	7.1	0.6	<DL	
	1000	<DL ³		64.5	16.9	8.1	0.5	<DL	
	500	<DL		53.8	6.6	6.4	1.0	<DL	
GM9	5000	5.1	3.3	>HS		11.2	0.3	4.7	1.0
	2500	3.5	1.4	>HS		10.0	0.3	3.0	0.6
	1000	4.5	1.0	45.3	15.1	10.5	0.8	<DL	
	500	<DL		51.0	27.8	<DL		<DL	
GM4N	5000	5.6	3.7	4.0	1.2	3.5	0.1	3.5	0.3
	2500	3.5	0.8	<DL		3.2	0.3	2.3	0.4
	1000	<DL		<DL		3.4	0.2	<DL	
	500	<DL		<DL		<DL		<DL	
GM1c9	5000	<0.5		>HS		0.9	0.1	<0.5	
	2500	<DL		>HS		<DL		<DL	
	1000	<DL		50.5	13.1	<DL		<DL	
	500	<DL		53.8	28.0	<DL		<DL	
GM1c	5000	1.4	0.3	>HS		5.8	0.3	1.0	0.6
	2500	1.7	0.3	>HS		5.4	0.4	<DL	
	100	<DL		>HS		5.0	0.2	<DL	
	500	<DL		128.8	9.6	<DL		<DL	
GM19	5000	<0.5		>HS		0.9	0.1	<0.5	
	2500	<DL		36.1	11.4	<DL		<DL	
	1000	<DL		32.6	4.8	<DL		<DL	
	500	<DL		26.0	7.6	<DL		<DL	

1. Results expressed as mean $\pm$ SD (n = 6)
2. For definitions of metabolite abbreviations see page 41.
3. Results < detection limit of the assay (DL)
4. Results > highest standards (HS)

Table III-2

Cross-reactivity of Cyclosporin G (CsG) metabolites in assays calibrated with CsG¹

Metabolite	Concentration ($\mu\text{g/L}$)	Sandimmune Specific		Sandimmune Non-specific		FPIA		Cyclotrac-SP	
		mean %	SD	mean %	SD	mean %	SD	mean %	SD
GM1 ²	5000	1.2	0.3	>HS ⁴		8.2	0.9	1.0	0.3
	2500	<DL ³		>HS		8.3	1.4	<DL	
	1000	<DL		>HSD		10.3	1.9	<DL	
	500	<DL		58.4	25.2	10.8	3.2	<DL	
GM9	5000	5.2	2.8	>HS		10.7	2.7	4.9	1.0
	2500	3.2	1.0	>HS		11.4	1.4	3.0	0.6
	1000	<DL		35.6	8.3	12.7	4.0	<DL	
	500	<DL		33.2	6.4	13.0	4.4	<DL	
GM4N	5000	2.6	1.0	5.5	2.7	3.6	0.3	3.2	0.3
	2500	2.1	0.7	<DL		4.2	0.8	2.3	0.4
	1000	<DL		<DL		5.2	1.3	<DL	
	500	<DL		<DL		<DL		<DL	
GM1c9	5000	<0.5		>HS		1.3	0.4	<0.5	
	2500	<DL		>HS		<DL		<DL	
	1000	<DL		42.4	15.0	<DL		<DL	
	500	<DL		31.0	8.4	<DL		<DL	
GM1c	5000	1.2	0.4	>HS		6.5	0.8	1.0	0.6
	2500	1.7	0.3	>HS		6.3	0.3	<DL	
	100	<DL		>HS		<DL		<DL	
	500	<DL		100.0	8.4	<DL		<DL	
GM19	5000	<0.5		>HS		1.3	0.5	<0.5	
	2500	<DL		24.8	4.6	<DL		<DL	
	1000	<DL		19.1	0.6	<DL		<DL	
	500	<DL		36.8	0.6	<DL		<DL	

1. Results expressed as mean $\pm$ SD (n = 6)

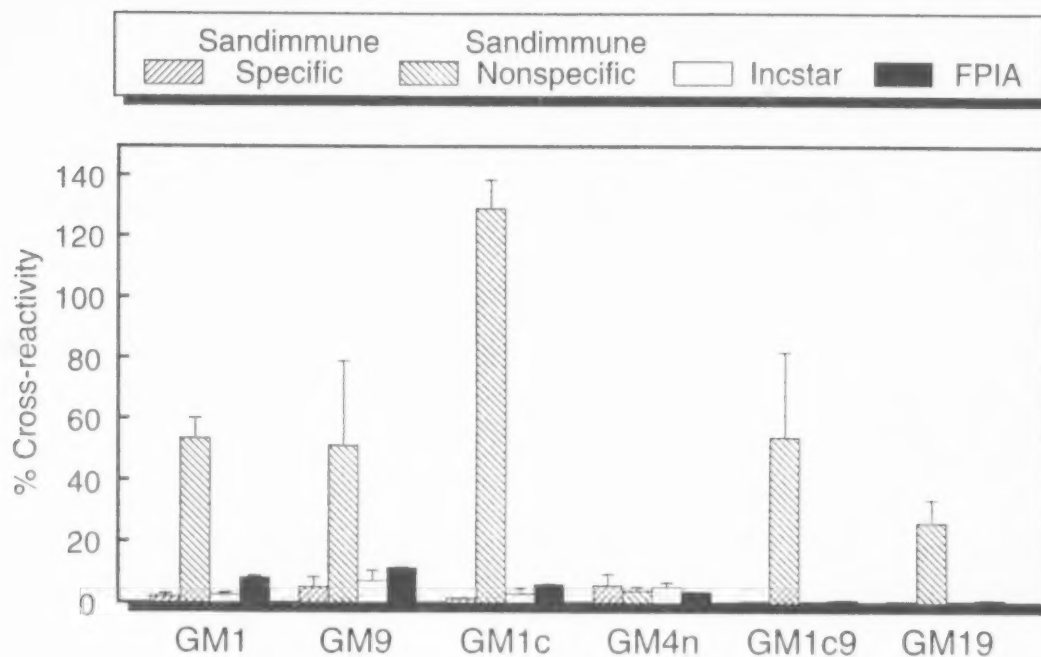
2. For definitions of metabolite abbreviations see page 41.

3. Results < detection limit of the assay (DL).

4. Results > highest standards (HS)

FIGURE III-2

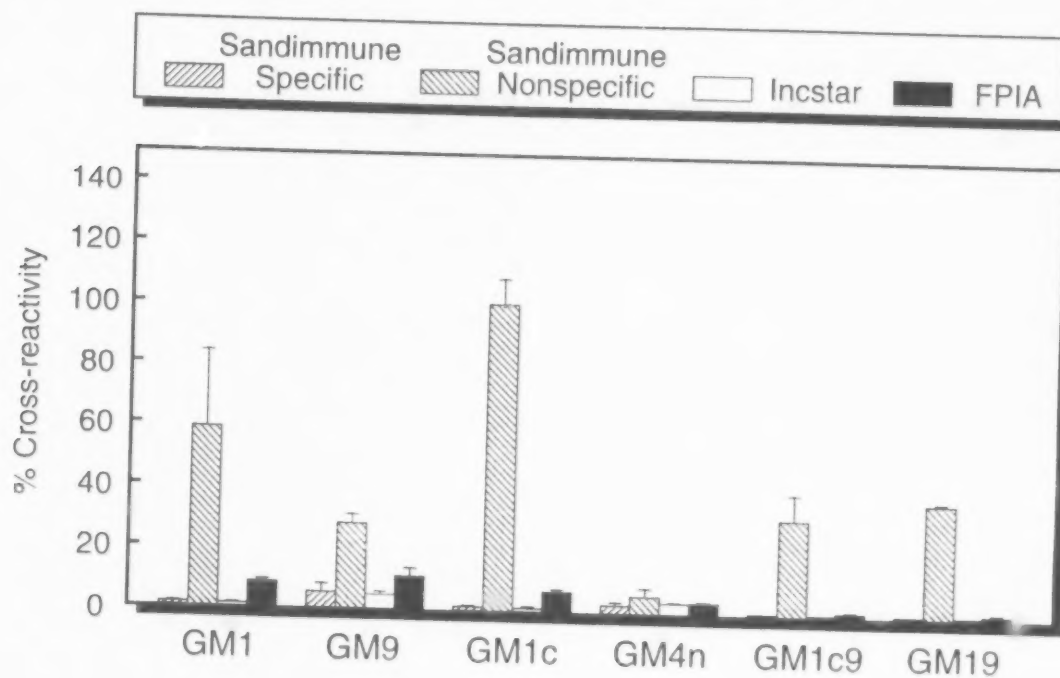
Cross-reactivities of Cyclosporin G (CsG) metabolites in assays calibrated with Cyclosporin A (CsA).



Cross-reactivities of CsG metabolites in assays calibrated with CsA. Cross-reactivities were determined at a metabolite concentration of 5000 $\mu\text{g/L}$ with the exception of the Sandimmune non-selective assay where a concentration of 500 $\mu\text{g/L}$ was used. Results expressed as $\bar{x} \pm \text{SD}$ ($n=6$). For definition of metabolites abbreviations see page 41.

FIGURE III-3

Cross-reactivities of Cyclosporin G (CsG) metabolites in assays calibrated with CsG.



Cross-reactivities were determined at concentrations described in Figure III-2. Results expressed as $\bar{x} \pm \text{SD}$ ($n=6$).

degree of the cross-reactivities for the assays investigated in ascending order was $SS = CT < FPIA < NS$.

There was no significant difference ($p > 0.05$) in the cross-reactivities of metabolites for the SS, FPIA and CT assay calibrated with either CsA or CsG (Tables III-1 and III-2, Figures III-2 and III-3). The difference in cross-reactivities for the assays calibrated with either standard at metabolite concentration of $5000 \mu\text{g/L}$ (ss, FPIA, and CT) and $500 \mu\text{g/L}$ (NS) are shown in Figure III-4. Results are expressed as the following difference: the cross-reactivity of metabolites in assays calibrated with CsA minus the cross-reactivity obtained when assays were calibrated with CsG.

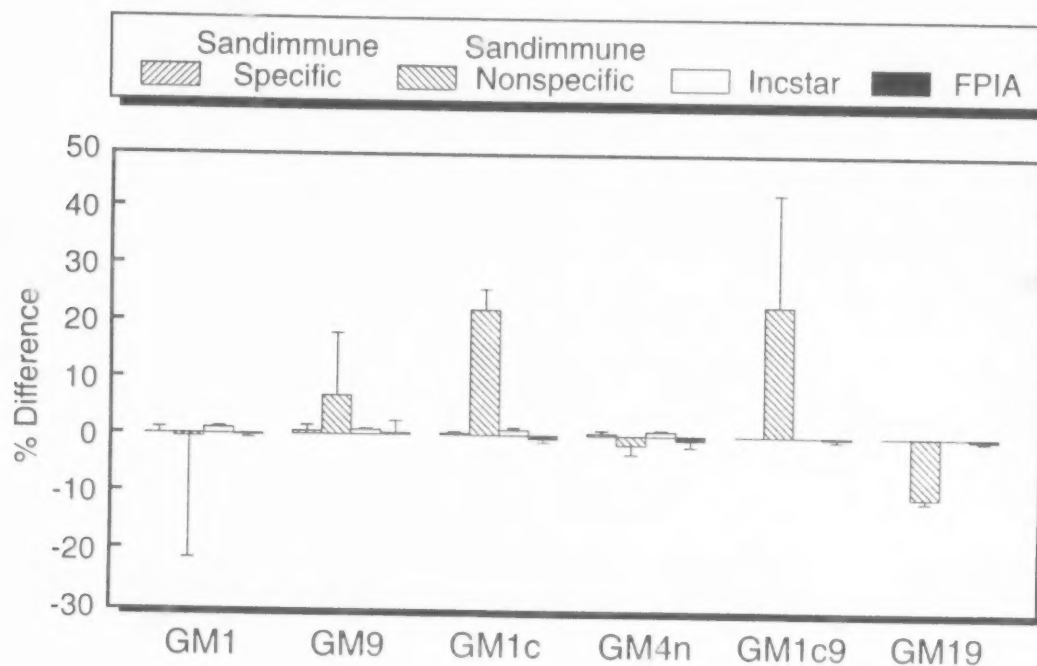
Cross-reactivities of metabolites in the presence and absence of $200 \mu\text{g/L}$ of CsG was determined for all assays. The differences between CsG metabolite cross-reactivities in each assay (CsA calibration) in the presence or absence of CsG at a metabolite concentration of $5000 \mu\text{g/L}$ ($500 \mu\text{g/L}$ for NS) are shown in Figure III-5. The data indicated that the cross-reactivities of the metabolites in the SS, FPIA and CT assay were similar whether parent CsG was present or not. In contrast, differences of up to 60% were noted with the NS assay.

4. DISCUSSION

In clinical studies the monitoring of the concentration of the drug in whole blood has been performed as a guide for dosage adjustment to maximize immunosuppressive efficacy and to minimize side-effects (63,64). The

FIGURE III-4

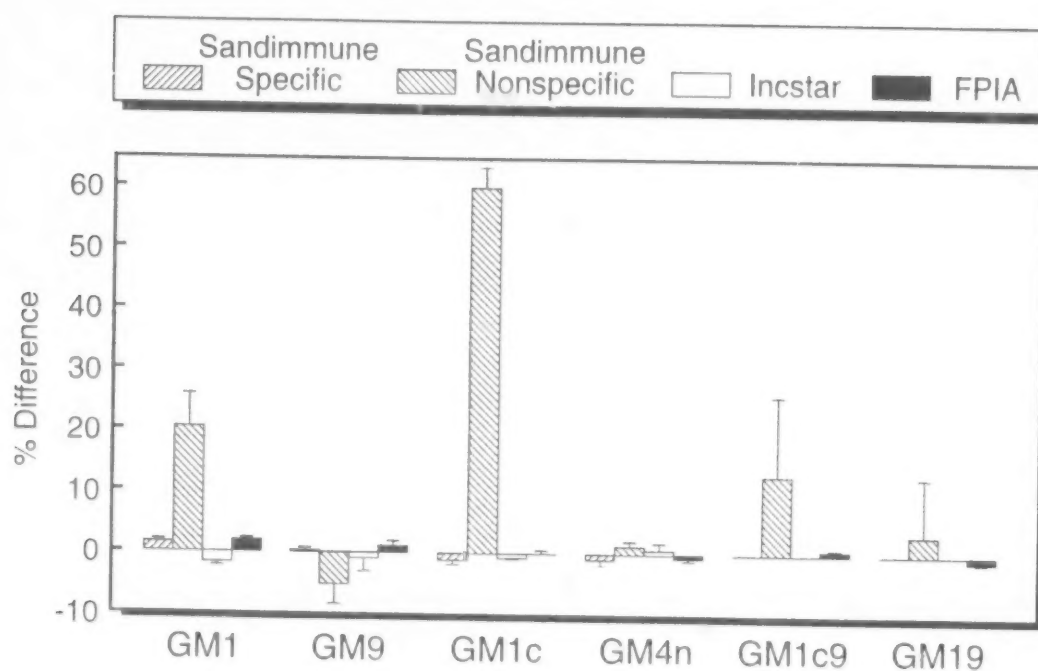
Differences in cross-reactivities of Cyclosporin G (CsG) metabolites in assays calibrated with Cyclosporin A (CsA) versus CsG.



Differences in cross-reactivities of CsG metabolites in assays calibrated with CsA versus CsG. Values were determined at a metabolites concentration of $5000\mu\text{g/L}$ for all assays except the Sandimmune non-specific where a concentration of $500\mu\text{g/L}$ was used. Results are expressed as the means ($\pm$ SD) difference of 6 determinations.

FIGURE III-5

Differences in cross-reactivities of Cyclosporin G (CsG) metabolites in assays in the presence and absence of (CsG).



Differences in cross-reactivities of CsG metabolites in assays in the presence and absence of CsG. All assays were calibrated with CsA standards. Values were determined at a metabolites concentration of $5000\mu\text{g/L}$ for all assays except the Sandimmune non-specific where a concentration of $500\mu\text{g/L}$ was used. Results are expressed as the mean ($\pm$ SD) difference of 6 determinations.

guidelines for the therapeutic monitoring of CsA stipulate that the drug should be measured in whole blood using assays that are selective for parent drug (191,192). Previous studies have indicated that the binding and distribution of CsG in whole blood is similar to that for CsA (55). CsG also appears to be metabolized in a manner similar to CsA (56-58). The structure and immunosuppressive activities of the metabolites of both drugs have been shown to be similar (56-58). This suggests that the guidelines for monitoring CsA would most likely be applicable for CsG. The measurement of CsG by HPLC would provide the most selective measurement of parent drug (190). However, due to the technical nature of HPLC, other less labour intensive methods must be investigated. A number of commercial immunoassays for the measurement of CsA are presently available (193,188,189,194). If these immunoassays are to be used for the measurement of CsG is it necessary to determine the cross-reactivities of CSG metabolites in these assays to ensure that the criteria for monitoring of the drug, as discussed above, are met.

CsG exhibited substantial ($\approx 100\%$) cross-reactivities with the SS, FPIA and CT assays. In contrast, cross-reactivities for the NS and EMIT assays were approximately 40% and 2% respectively. This is due to the epitopes of the molecule against which the monoclonal antibodies are directed (195,196). Selective monoclonals used in the SS, CT, and FPIA assays all recognize an epitope which does not include the amino acid 2 region of the molecule, the residue in which the difference between CsA and CsG resides. In contrast, the

NS and EMIT assays must recognize an epitope which includes the amino acid 2 region of the molecule and thus exhibit less cross-reactivity (195,196). Due to the minimal cross-reactivity of CsG in the EMIT assay, further evaluation of this assay with CsG metabolites was not performed.

The concentrations of CsG metabolites used in this study span the expected range *in vivo* based on previous studies with CsA (57). The NS assay exhibited the most cross-reactivity with CsG metabolites. Less cross-reactivity was found with the SS, FPIA and CT assays. Metabolites with a single modification (GM9, GM4N, and GM1), exhibited the most cross-reactivity in such assays. The cross-reactivities with the secondary metabolites (GM1c, GM19, GM1c9) were generally substantially less. The maximal cross-reactivities of these metabolites in the specific assays (SS, FPIA, and CT) were > 12%. The cross-reactivities with the secondary metabolites (GM1c, GM19, GM1c9) were generally less. The SS and CT assays were found to be the most selective for parent drug followed by the FPIA and NS assays. These results are consistent with the cross-reactivities of these assays previously reported for CsA metabolites (188,189,191-193,197,198).

Specimens obtained from patients receiving CsG will contain parent drug as well as metabolites. To determine whether the presence of CsG would affect the cross-reactivity of its metabolites over a concentration range of 500-5000 $\mu\text{g/L}$, a comparison of the cross-reactivities in the presence and absence of parent drug was performed. A concentration of parent drug was chosen

(200 $\mu\text{g/L}$) which would be well within the blood concentration range observed for the drug when immunosuppressive doses are administered (63,64). The results indicate parent drug had no effect on the cross-reactivities of metabolites in the SS, FPIA and CT assays. This is expected for the specific assays since the monoclonal antibody is added in excess, as compared to the analyte (CsA/CsG), to the assay system, and the amount of parent CsG is in a relatively low concentration. In contrast, in the NS assays the presence of CsG resulted in a substantial increase in cross-reactivity with some metabolites. In this case the presence of CsG might, by unknown mechanisms, alters the affinity of the non-selective monoclonal antibody used in the NS assay for metabolites.

For laboratories required to monitor both CsA and CsG, it would be advantageous to be able to measure both drugs with a single assay. We have shown that the cross-reactivities of metabolites for the SS, FPIA and CT assays are similar in assays calibrated with CsG or CsA. The results suggest that these assays can be used for monitoring of CsG. The EMIT assay which exhibits minimal cross-reactivity with CsG could not be used for monitoring of the drug. From a clinical perspective, the routine use of a correction factor to account for the cross-reactivity of CsG in assays calibrated with CsA most likely will not be necessary, since the difference in values is small and well within the range of analytical variation of the assays.

The results presented here indicate that the routine monitoring of CsG

can be performed with commercial immunoassays available for the monitoring of CsA. However, cross-reactivities with CsG metabolites does occur to a varying extent with all assays tested. The significance of these cross-reactivities in the routine monitoring of CsG has not been determined. The measurement of steady state concentrations of metabolites in transplant patients is required to assess this, and will be discussed in Chapter IV.

IV. STEADY STATE CONCENTRATIONS OF CYCLOSPORIN G (CsG) METABOLITES IN RENAL TRANSPLANT PATIENTS

1. RATIONALE:

The impact of CsG metabolites on the monitoring of the drug is not only dependent on the cross-reactivities as determined but also on their steady state concentrations. While the cross-reactivities of CsG metabolites in immunoassays were reported in Chapter III, the steady state concentrations of the metabolites in transplant patients are not known. The proportion of parent CsG in relation to total drug measured in these assays is also unknown. Such information would provide a framework for the interpretation of CsG blood concentrations and for the assay of specific dosage modifications. The steady state concentrations of CsG and six of its metabolites throughout the 12 h dosing interval in renal transplant recipients was therefore investigated.

2. MATERIALS AND METHODS

a. Source of drugs and metabolites

CsA and CsG were provided as gifts from Sandoz Inc. (East Hanover NJ). CsG metabolites GM1, GM9, GM4N, GM1c, GM1c9, and GM19, were isolated in our laboratory by HPLC from urine from patients receiving the drug as previously described in Chapter III. Structure and purity were confirmed by FAB-MS and ^{13}C -NMR and the purity of all metabolites was determined to be

≥97%.

b. Patient samples

Six patients enrolled in the CsG treatment arm of a clinical trial comparing the safety and efficacy of CsG to that of CsA in the prophylaxis of acute cellular rejection were utilized in this study. Each received an oral solution of CsG in place of CsA in addition to AZA and prednisone. Total daily doses of CsG ranged from 220-660 mg/day. All subjects were at least 6 months post transplant, had stable renal function, were clinically free of rejection, and had not undergone dosage adjustments of any of their medication in the two weeks prior to study enrollment. Agents known to alter CsA or CsG levels were not co-prescribed.

On day 1 of the study, after an overnight fast, a pre-dose specimen was obtained. The patients customary dose of CsG was administered, and post-dose specimens were collected at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0 and 12.0 h. The following day (day 2) the subjects received a moderate fat breakfast, with the procedure described above being repeated. To determine whether the type of formulation would alter the metabolism of the drug, the next morning (day 3), patients were switched, on a mg to mg basis, to a soft gelatin capsule form of CsG and treated with the new formulation for seven days, until steady state was reattained. The day 1 and 2 sampling procedures were then repeated as described above. In total, 4 sets of specimens were collected from each patient. All specimens were collected in tubes containing

EDTA and stored at -70°C until analysis. CsG and its metabolites have been shown to be stable for up to 1 year under these storage conditions (unpublished observations). All specimens were analyzed prior to this time.

c. Metabolite concentration determination

CsG and its metabolites were measured in whole blood using a modification of a liquid extraction procedure previously described ([193](#)). Chromatographic separation was achieved with an isocratic system with flow rate at 1.0 mL/min using a reverse-phase C_8 column with a mobile phase of acetonitrile/methanol/ H_2O (42/20/38). The solvent flow rate was 1.0 mL/min with the column temperature maintained at 70°C . The eluted peaks were monitored at 214 nm. The concentration was calculated from the area under the curve in relation to an CsA internal standard. The between-run coefficients of variation for the method were $< 12\%$ at $100\text{ }\mu\text{g/L}$ and $< 9\%$ at $400\text{ }\mu\text{g/L}$, for CsG and the 6 metabolites measured.

d. Immunoassays

The concentration of CsG was determined using two monoclonal immunoassays originally developed for the measurement of CsA: a FPIA procedure (TDx Abbott Diagnostics, Abbott Park IL) and a RIA procedure (Cyclotrac-SP Incstar Inc., Stillwater, MN) as described in Chapter III. All reagents were included in the kits. The assays were performed according to the manufacturers' instructions using whole blood specimens ([189,193](#)). The inter-assay coefficients of variation at CsG concentrations between 100 and

400 $\mu\text{g/L}$ were $<7\%$ for the FPIA and $<10\%$ for RIA.

3. RESULTS

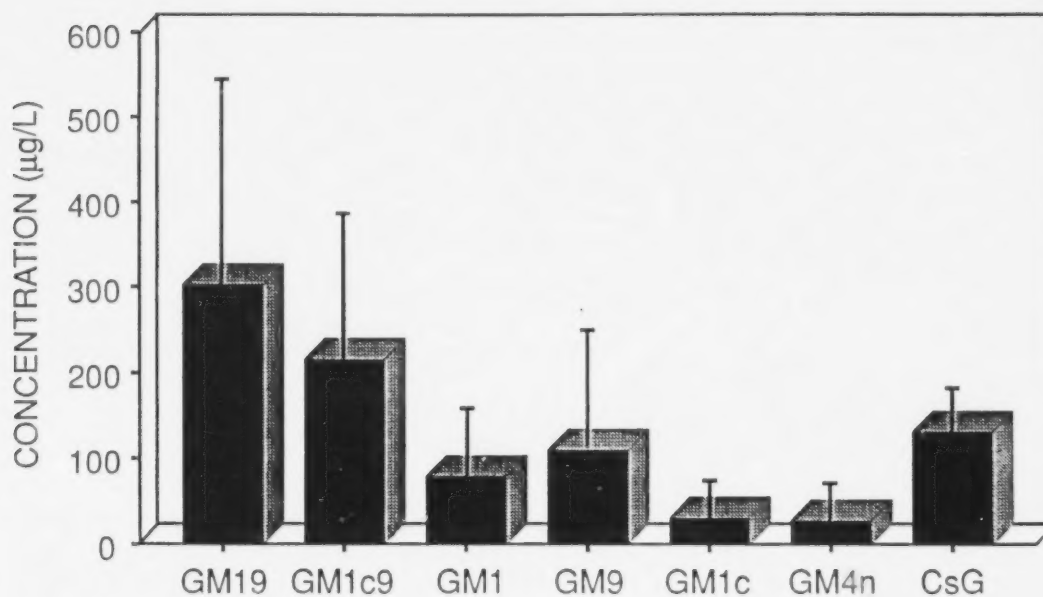
a. Concentration of metabolites

The steady state 12 hour trough concentrations of CsG and six of the major metabolites are shown in Figure IV-1. The data from both phases were combined since no statistical difference ($p>0.05$) was found in the concentration of CsG or its metabolites between the treatment phases for each individual patient. At steady state, the secondary metabolites GM19 and GM1c9 are present at a mean trough concentration of 2-3 times that of the parent CsG. The other metabolites are present in lower mean trough concentrations relative to the parent drug (Table IV-1).

The mean concentrations of parent CsG and metabolites over the dosing interval are shown in Figure IV-2A and IV-2B. Through most of the dosing interval parent CsG is present in the highest concentration. The primary metabolites (GM1, GM9, GM4N) have a mean concentration that is lower than that of the parent drug. The relative proportion of these metabolites is generally maintained throughout the dosing interval. This trend was not observed for the secondary metabolites. GM19 and GM1c9 show an increase in relative as well as absolute concentrations commencing 8-10 h post-dose. At 12 h post-dose, these metabolites have a mean concentration that is higher than that of the parent CsG. GM1c shows very little change in mean

FIGURE IV-1

Twelve hour trough concentrations of Cyclosporin G (CsG) and metabolites in 6 renal transplant recipients.



Twelve hour trough concentration of CsG and metabolites in 6 renal transplant recipients. A total of 4 specimens were collected from each patient. Results are expressed as mean $\pm$ SD (n = 24).

Table IV-1

Comparison of mean relative steady state 12 hour trough concentrations of CsG and CsA^a metabolites^b

CsA ^a	%	CsG	%
CsA	100	CsG	100
AM1 ^c	150	GM1 ^d	63
AM9	75	GM9	81
AM4N	25	GM4N	17
AM1c	40	GM1c	16
AM1c9	5	GM1c9	165
AM19	69	GM19	231

a data taken from references 189,193,199.

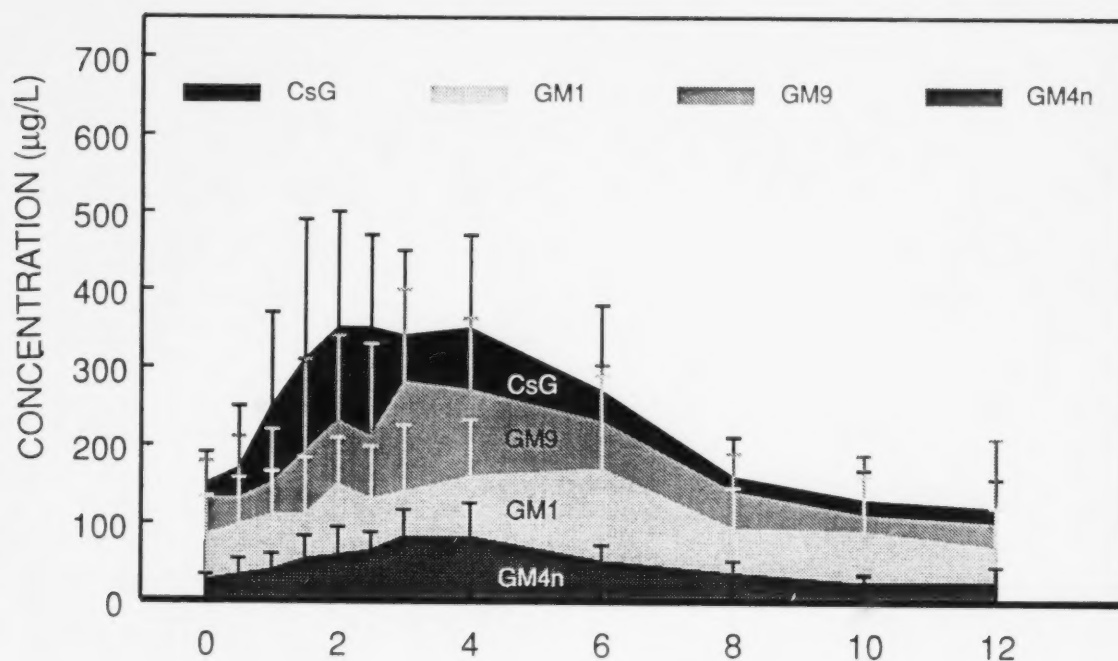
b results expressed as a % of parent drug concentration.

c CsA metabolite 1 (refer to page 41)

d CsG metabolite 1 (refer to page 41)

FIGURE IV-2A

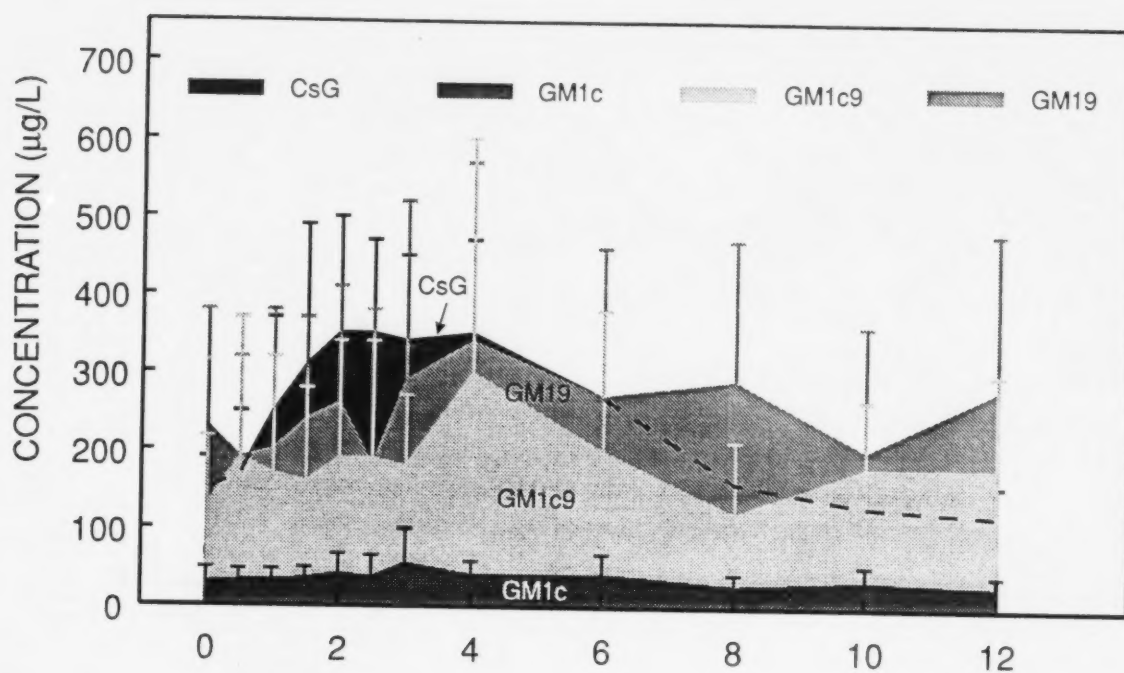
Concentration of Cyclosporin G (CsG) and its primary metabolites



Concentration of CsG and its primary metabolites (A) over a 12 hour sampling interval in renal transplant recipients. A total of 4 specimens were collected from each patient. Results expressed as mean $\pm$ SD ($n=24$).

FIGURE IV-2B

Concentration of Cyclosporin G (CsG) and its secondary metabolites



Concentration CsG and its secondary metabolites (B) over a 12 hour sampling interval in renal transplant recipients. A total of 4 specimens were collected from each patient. Results expressed as mean $\pm$ SD (n = 24).

concentration over the dosing interval whereas, GM19 and GM1c9 show extensive variation over the dosing interval as indicated by the large SD. A comparison of the steady state 12 hour trough concentrations of CsG and metabolites to those previously reported for CsA is shown in Table IV-1.

b. Correlation studies

The steady state 12 hour trough values obtained for CsG by FPIA and RIA were compared to those obtained by HPLC. The following relationship was observed: FPIA (y) versus HPLC (x), $y = 1.15x + 10.82$, $r = 0.87$, $Sy/x = 49.8$, $n = 24$; RIA (y) versus HPLC (x), $y = 1.33x + 18.4$, $r = 0.82$, $Sy/x = 33.9$, $n = 24$. The mean bias of FPIA and RIA versus HPLC was 19 and 36%, respectively. The FPIA/HPLC ratios for individual patients ranged from 1.05 - 1.54, while that for RIA/HPLC was 1.25 - 1.80. To determine whether the biases in the immunoassays could be accounted for by the cross reactivity of the metabolites, the estimated bias for each assay was determined from the sum of the products of the cross-reactivity for each metabolite, as previously determined in Chapter III and updated in a few instances due to further experimental validation in the present study, multiplied by the steady state trough concentration for each metabolite as determined by HPLC. The calculations, which are shown in Table IV-2, indicated that the calculated biases for the FPIA and RIA procedures of 19 and 28% were not significantly different from the observed bias as reported above.

4. DISCUSSION

The data presented here indicate that the trough concentrations of the primary metabolites of CsG are lower, and those of the secondary metabolites, GM19 and GM1c9, are higher than the trough concentrations of parent drug. This is in contrast to CsA, in renal transplant recipients, where the primary metabolite AM1 has been reported to be present at a higher concentration (167-169,200) and the secondary metabolites AM19 and AM1c9 in lower concentrations than parent CsA. These data suggest that the metabolism of primary metabolites to secondary metabolites occurs more extensively with CsG than with CsA. The rise in GM19 and GM1c9 near the end of the dosing interval is most likely due to their conversion from primary metabolites, and may be amplified by enterohepatic circulation. The latter has also been suggested for some of the CsA metabolites (57). It should be noted that only 6 stable renal transplant recipients were included in the study. It is presently not known whether the data will be applicable to other transplant categories.

In clinical studies, therapeutic monitoring of the concentration of the drug in whole blood has been useful as a guide for dosage adjustment (201,59). Specimens obtained from patients receiving CsG will contain parent drug as well as metabolites. To determine the contribution of CsG metabolites to the measurement of the drug by immunoassay procedures, the steady state 12 h trough concentrations of CsG and six of its metabolites were determined. By utilizing the known cross reactivities of parent CsG and its metabolites, and the

corresponding 12 h trough concentrations, the contribution that the metabolites have on the analytical bias was calculated. It was found that the estimated biases versus HPLC were 19 and 28%, for the FPIA and RIA assays respectively, which compares well with the observed biases of 19 and 36%. Therefore, the difference between the HPLC result, when parent is measured, and the immunoassay result is accounted for, in large part, by the cross reactivity of the metabolites. The inter-individual variation in the FPIA/HPLC and RIA/HPLC ratios most likely indicates differences in metabolite concentrations from patient to patient. Annesley et al. have reported a bias of 37% for RIA versus HPLC which is consistent with the results reported here (202). However the bias they reported for FPIA versus HPLC at 38% was significantly greater (202). This may result from differences in the patient population studied.

In this study, only 6 of the 7 metabolites of CsG which have been isolated and characterised were measured (56). However, as CsG is metabolised in a manner similar to CsA, there are possibly more than 20 metabolites of the former drug (57). As the majority of the FPIA/HPLC and RIA/HPLC bias could be accounted for by the 6 metabolites measured, this suggests that the remaining metabolites are either present in very low concentrations and/or their cross-reactivities in the assays are minimal.

However, the contribution of metabolites to test results varies with the assays employed. The results here suggest that the steady-state

concentrations of metabolites of CsG and CsA differ. This difference in concentration and cross-reactivity leads to greater biases in immunoassays used to measure the drug. Appropriate therapeutic range adjustments would be required to compensate for any bias that metabolite cross reactivities may contribute to the assay value. The utility of these assays for the routine monitoring of CsG needs to be confirmed by clinical trials.

V. *IN VITRO* TOXICITY OF CsG METABOLITES

1. RATIONALE:

CsA induced renal changes are the major side-effect of drug therapy. There is evidence to suggest that the pathogenesis of CsA renal side-effects involves alterations in the release of vasoconstrictive substances and/or vasodilatory prostanoids (17,48). The effect of CsA and its metabolites on the release of prostacyclin (PGI₂) and endothelin-1 (ET-1) production in primary cultures of rabbit mesangial cells has previously been investigated (203). The results indicate that some CsA metabolites are more potent than parent drug in the release of ET-1. This suggests that CsA metabolites may contribute to the pathogenesis of CsA induced renal side-effects. However, little is known regarding the toxicity of CsG metabolites. As CsG induces fewer renal side-effects than CsA, this may be related to a decreased effect the former may have on the release of vasoactive substances. The effect of CsG and its metabolites in comparison to CsA and its metabolites on the release of ET-1 and PGI₂ from primary renal cell lines was investigated.

2. MATERIALS AND METHODS

a. Source of drugs and metabolites

CsA and CsG were provided as gifts from Sandoz Inc. (East Hanover NJ). CsG and CsA metabolites were isolated by diethyl ether extraction and HPLC

from urine from renal transplant patients receiving either drug, as previously described in Chapter I. The structure and purity of the metabolites were confirmed by fast atom bombardment mass spectrometry (FAB-MS) and ^3H - and ^{13}C -nuclear magnetic resonance (NMR). In all instances the metabolites were >97% pure. The CsG metabolites examined were GM1, GM9, GM4N, GM1c, GM1c9, and GM19, while those for CsA were AM1, AM9, AM4N, AM1c, AM1c9, and AM19.

b. Isolation of rabbit mesangial cells

Primary cultures of mesangial cells were obtained from collagenase treated isolated rabbit glomeruli. Whenever possible, aseptic techniques were used. Briefly, the kidneys from a New Zealand white rabbit were removed and placed in sterile Hanks' Balanced salt solution (HBSS) containing 25 mM Hepes, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (all from Gibco Laboratories, Burlington, ON, Canada). The kidney was sliced into thin sections and the outer cortex removed. The glomeruli were isolated from the cortex using a differential sieving technique as previously described (19,24). Briefly, the isolated glomeruli were washed twice with HBSS and then incubated with trypsin (0.2% w/v) for 20 min. at 37°C. This was followed by incubation with type IV collagenase (0.1% w/v) (Sigma Chemical Co. St. Louis. MO). The collagenase treated glomeruli were washed once with HBSS and resuspended in the medium and plated. Microscopic examination revealed the glomeruli were > 95% free of tubular contamination. The growth medium used was

Dulbecco's minimal essential medium (DMEM) supplemented with 20% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, 1.0% (v/v) non-essential amino acids (all from Gibco Laboratories), and 0.66 U/mL protamine zinc insulin (Novo Laboratories Ltd., Willodale, ON, Canada). After 10 days, subclones of individual cell outgrowths were removed by treatment with trypsin (0.05% w/v) and EDTA (0.02% w/v) and replated. The cells were grown for three passages in a medium in which L-valine was replaced with D-valine to prevent growth of fibroblasts. The latter cells are unable to proliferate in the latter medium since they lack the enzyme D-amino acid oxidase (204). Microscopic examination of the culture revealed a homogeneous population of spindle shaped cells. These cells were subsequently passed every seven days in DMEM without antibiotics, since it has been previously shown that the latter may act in synergy with CsA in its toxic effect on such cells (58,205). After six passages the cells were used for the subsequent experiments as described below.

c. Isolation of rabbit endothelial cells

Primary cultures of endothelial cells were obtained from collagenase treated rabbit aorta using a modification of a procedure previously described (206). Whenever possible, aseptic techniques were used. Briefly, the thoracic aorta from arch to diaphragm was removed from New Zealand white rabbits and placed in sterile Hanks' Balanced salt solution (HBSS) containing 25mM Hepes, 100 U/mL penicillin, and 100 μ g/mL streptomycin (all from Gibco

Laboratories, Burlington, ON, Canada). After it was trimmed of fat, each aorta was cut lengthwise with scissors to expose the intima, which was irrigated with HBSS solution to wash away the remaining blood. The aorta was placed intima side down in a puddle of sterile collagenase solution. After 30 min. incubation at 37°C the aorta was reinserted in a fresh dish without enzyme and the intimal surface was scraped with a scalpel. Scrapings were collected after each unidirectional stroke. The collagenase treated cells were washed once with growth medium, resuspended and plated. The growth medium used was M-199 20% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1.0% (v/v) non-essential amino acids (all from Gibco Laboratories), 25 mM Hepes, 50 mg/L ascorbic acid, 2 mM glutamine, and 0.66 U/mL protamine zinc insulin (Novo Laboratories Ltd., Willodale, ON, Canada). Microscopic examination of the cultures revealed a homogenous population of polygonal cells positive for Factor VIII antigen. The cells were passed in medium devoid of antibiotics prior to experimentation for the reasons described above.

d. Effect of CsG and metabolites on rabbit mesangial and endothelial cell growth and DNA synthesis.

The cells were plated at a density of 2×10^4 cells per 9 cm² six-well tissue culture plates. CsG, CsG metabolites, or the vehicle control, were added at final concentrations up to 25000 µg/L. The vehicle consisted of 70:30 ethanol/Tween 80 (v/v). The final ethanol concentration for all experiments

was $\leq 0.2\%$ (v/v), which did not affect cell growth or DNA synthesis. Five days after plating the cells were recovered by trypsinization, washed with a growth medium, washed with saline, and counted using a haemocytometer. A portion of the cells was analyzed for protein content. DNA synthesis was determined as previously described by measuring the incorporation of 2 μCi [^3H]-thymidine into TCA insoluble macromolecules 18 h prior to trypsinization (173). Each experiment was done in duplicate and repeated on three separate days ($n = 6$) for each concentration of the drug or metabolite. All values were corrected for protein concentration and the concentration required for 50% inhibition of cell growth and DNA synthesis (IC_{50}) of cell growth on DNA synthesis was determined. Results were also expressed as potency ratio relative to CsG

$$\text{potency ratio} = \frac{\text{IC}_{50} \text{ CsG}}{\text{IC}_{50} \text{ metabolite}}$$

e. Effect of CsG and metabolites on the release of endothelin

The basal release of ET-1 from cultured mesangial and endothelial cells was determined by using a modification of a procedure previously described (207). Briefly, the medium was removed from confluent mesangial and endothelial cells grown in 9-cm² six-well tissue culture plates. The cells were washed twice with sterile saline. CsG and individual metabolites were added to serum free medium to a final concentration of either 500 or 5000 $\mu\text{g/L}$. Subsequent to incubation at 37°C in 5% CO_2 , the medium was removed at

intervals of 1, 6 and 24 h and stored at -70°C until analysis. ET-1 was determined by RIA as described by the manufacturer (Amersham Canada Ltd). All experiments were performed on at least three different days ($n = 3$). The cells from each well were removed by treatment with trypsin and the protein content determined as described above. The final results were expressed as fmol ET-1/mg protein and expressed as a mean $\pm$ SD. Comparison between the means of the groups was done using the Student's *t* test. A *p* value of <0.05 was considered significant.

f. Effect of CsG and metabolites on the release of prostacyclin

The basal release of 6-ketoprostaglandin $\text{F}_{1\alpha}$ (6-keto-PGF $_{1\alpha}$), the stable metabolite of PGI $_2$, from mesangial and endothelial cells was measured in the presence of CsG and its metabolites or vehicle using the procedure described above. The samples were stored at -70°C until analysis. Samples were diluted from 1:1 to 1:9 with saline and the amount of 6-keto-PGF $_{1\alpha}$ was determined by RIA (Amersham Canada Ltd). All experiments were repeated on at least three different days ($n = 3$). The protein content was determined as described above and the final results were expressed as pg 6-keto-PGF $_{1\alpha}$ /mg protein. All values were expressed as mean $\pm$ SD. Comparison between the means of the groups was performed as described above. A *p* value of <0.05 was considered significant.

g. Comparison of the effects of CsA, CsG and their metabolites on the release of endothelin and prostacyclin

To compare the effects that CsA and CsG have on the release of vasoactive substances from primary renal cell lines, cells were incubated in the presence of parent drug in combination with their metabolites. Two concentrations of parent drug, 200 and 500 $\mu\text{g/L}$, were investigated since they span the ranges encountered clinically. The concentrations of metabolites in relation to parent drug were chosen based on their relative trough concentrations observed at steady state as described in Chapter IV as shown in Table V-1. The combination of parent drug and metabolites was then incubated with both cell lines and the release of vasoactive substance was measured as described above.

3. RESULTS

a. Effect of CsG metabolites on cell growth and DNA synthesis

The effect of various concentrations of CsG and metabolites on cell growth and DNA synthesis in rabbit and mesangial and endothelial cells was investigated and the results shown in Table V-2. The concentration of CsG or metabolites that resulted in a 50% inhibition of these parameters was determined. The IC_{50} values of CsG for inhibition of cell growth and DNA synthesis for endothelial cells was 6667 ± 1178 and $11250 \pm 1250 \mu\text{g/L}$, respectively. In contrast, the IC_{50} values for mesangial cells were 3667 ± 1886 and $5000 \pm 0 \mu\text{g/L}$, respectively. Metabolite GM9 was the most potent having an IC_{50} of $20833 \pm 5892 \mu\text{g/L}$, with a potency relative to parent CsG

Table V-1

Concentration of Cyclosporin A, Cyclosporin G and their metabolites used to investigate the Release of Endothelin and Prostacyclin from Rabbit Renal Cell Lines.*

	$\mu\text{g/L}$			$\mu\text{g/L}$	
CsA	200	500	CsG	200	500
AM1	300	750	GM1	126	315
AM9	150	375	GM9	162	405
AM4N	50	75	GM4N	34	85
AM1c	80	100	GM1c	32	80
AM1c9	10	25	GM1c9	330	825
AM19	138	345	GM19	462	1155
* Relative concentration of metabolites in relation to parent drug taken from references 57,167-166, Chapter IV.					

Table V-2

The effect of Cyclosporin G (CsG) and its metabolites on cell growth and DNA synthesis in rabbit mesangial and endothelial cells (n = 6).

Cell Growth metabolite	Mesangial cells		Endothelial cells	
	IC ₅₀ mean	SD	IC ₅₀ mean	SD
CsG	3666	1885	6667	1179
GM1	> 25000	N/A ¹	25000	N/A
GM9	20000	7071	20833	5892
GM4N	> 25000	N/A	> 25000	N/A
GM19	> 25000	N/A	> 25000	N/A
GM1c9	> 25000	N/A	> 25000	N/A
GN1c	> 25000	N/A	> 25000	N/A
DNA Synthesis				
metabolites	mean	SD	mean	SD
CsG	5000	N/A	11250	1250
GM1	> 25000	N/A	> 25000	N/A
GM9	> 25000	N/A	> 25000	N/A
GM4N	> 25000	N/A	> 25000	N/A
GM19	> 25000	N/A	> 25000	N/A
GM1c9	> 25000	N/A	> 25000	N/A
GM1c	> 25000	N/A	> 25000	N/A

1 - not available

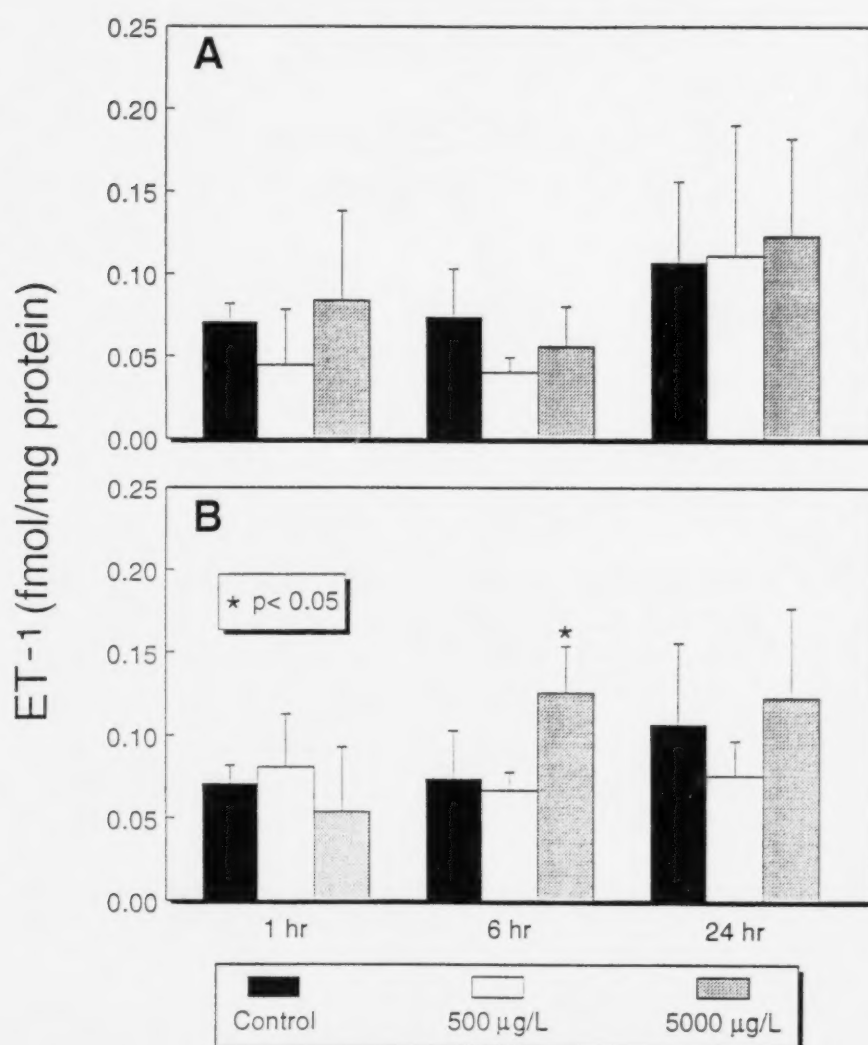
(potency ratio of 0.32), for inhibition of endothelial cell growth and an IC_{50} of $20000 \pm 7071 \mu\text{g/L}$, (potency ratio of 0.18), for inhibition of mesangial cell growth. GM9 did not exhibit an effect on DNA synthesis at a concentration of $25000 \mu\text{g/L}$, the highest concentrations tested, for either cell line. The remaining metabolites were found to exhibit minimal effects on cell growth or DNA synthesis at $25000 \mu\text{g/L}$ for both cell lines.

b. Effect of CsG and metabolites on the release of endothelin and prostacyclin

The effect of CsG and metabolites on the release of ET-1 and PGI_2 from mesangial and endothelial cells was examined. There was no significant difference between the media control and the vehicle control. CsG at concentrations and times investigated did not result in a significant change as compared to control cultures on the release of ET-1 (Figure V-1A) or PGI_2 (Figure V-2A) for both cell types. However, the metabolite GM1 at a concentration of $5000 \mu\text{g/L}$ resulted in a significant ($p < 0.05$) increase in ET-1 release after 6 hours of incubation with mesangial cells (Figure V-1B). The remaining metabolites did not show any statistically significant alteration in the release of ET-1 at any time point or at any concentration for both cell lines. Metabolite GM9 at concentrations of 500 and $5000 \mu\text{g/L}$ resulted in a significant decrease in PGI_2 release after 24 hours of incubation with mesangial cells (Figure V-2B), while the remaining metabolites did not significantly affect the release of this analyte at any time point or any concentration.

FIGURE V-1

The effect of Cyclosporin G (CsG) and metabolite GM1 on the release of endothelin by mesangial cells.

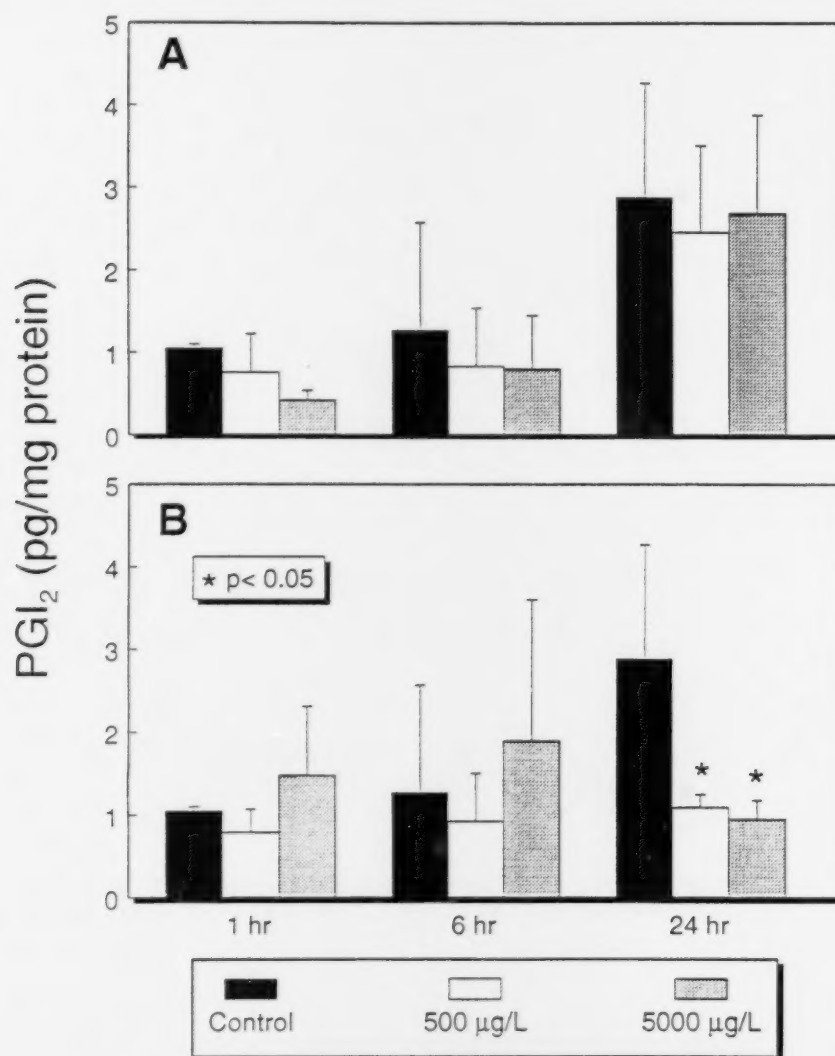


* $p < 0.05$ as compared to control cultures

The effect of CsG (A) and metabolite GM1 (B) on the release of endothelin by mesangial cells. Results are expressed as mean $\pm$ SD ($n = 3$).

FIGURE V-2

The effect of Cyclosporin G (CsG) and metabolite GM9 on the release of prostacyclin by mesangial cells



The effect of CsG (A) and metabolite GM9 (B) on the release of prostacyclin by mesangial cells. results are expressed as mean $\pm$ SD (n = 3).

c. **Comparison of CsA, CsG and their metabolites on the release of endothelin and prostacyclin.**

The cell cultures were also treated with parent drug (CsA or CsG) in combination with the metabolites at concentrations consistent with trough levels observed at steady state as shown in Table V-1, followed by measurement of the release of ET-1 and PGI₂. CsA and CsG in combination with their respective metabolites did not result in a statistically significant alteration in the release of PGI₂ at any time point or concentration with both cell lines (Figure V-3A and V-3B). The release of ET-1 by mesangial cells was also unaffected by CsA and CsG in combination with their metabolites (Figure V-4A). In contrast, the release of ET-1 by endothelial cells was statistically higher ($p < 0.05$) with both the CsA and CsG in combination with their metabolites at both concentrations tested (Figure V-4B).

4. **DISCUSSION**

Previous *in vitro* data with primary renal cell lines suggests a possible role of CsA metabolites in CsA induced renal side-effects (203). However, similar data investigating CsG and its metabolites has not been reported. In this study, the effect of CsG and its metabolites on primary rabbit endothelial and mesangial cells was investigated. The concentration of CsG resulting in a 50% inhibition of cell growth and DNA synthesis was similar to previous results obtained for CsA (203,208). GM9 was the most potent CsG metabolite

FIGURE V-3

The effect of Cyclosporin G (CsG), Cyclosporin A (CsA), in combination with their respective metabolites on the release of prostacyclin by mesangial cells (A), and endothelial cells (B). The relative concentrations of metabolites in relation to parent drug are based on trough steady state levels. Results are expressed as mean $\pm$ SD ($n=3$).

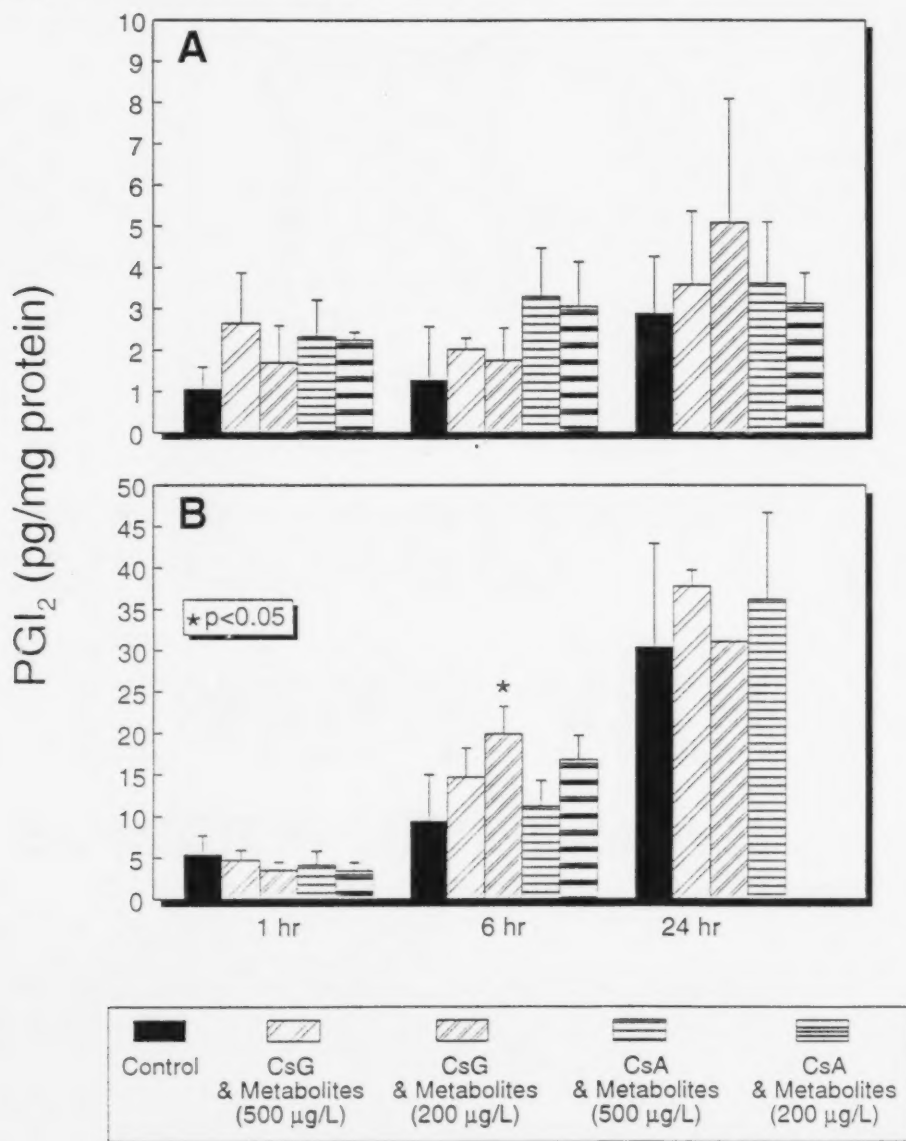
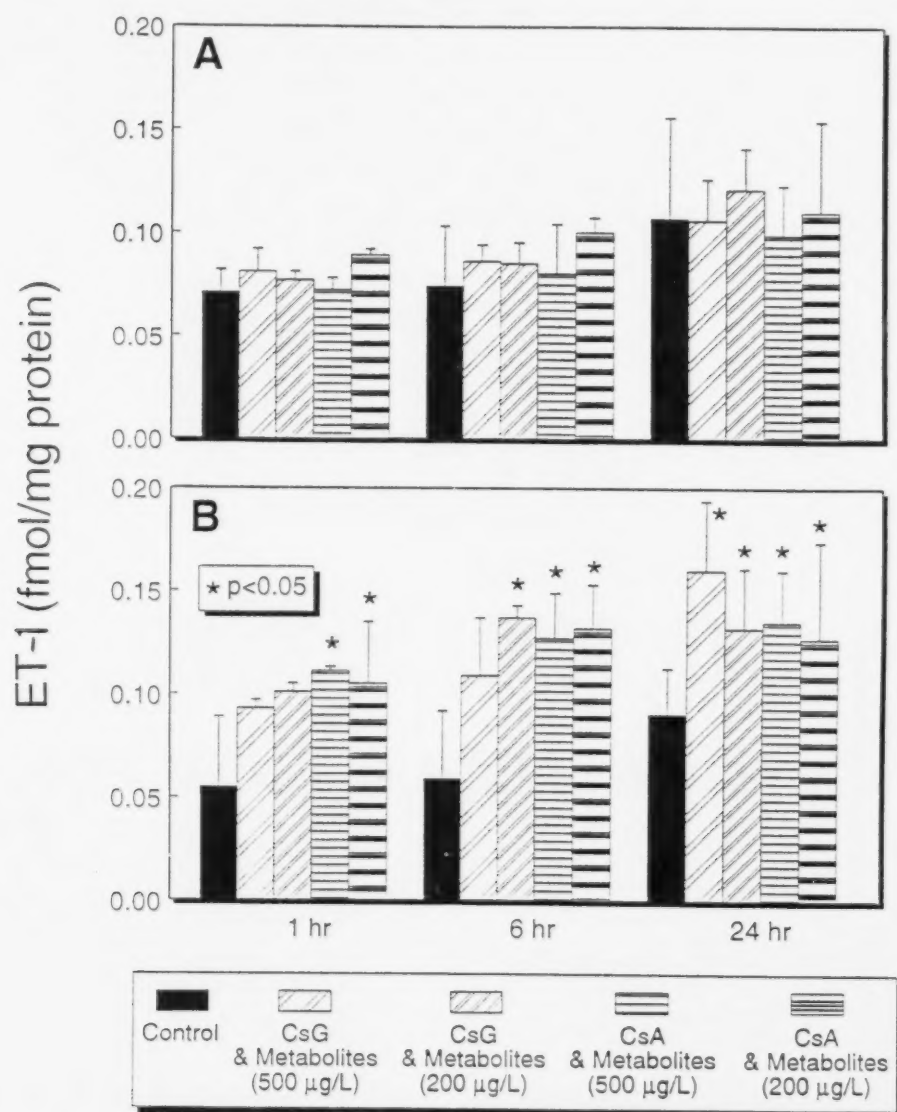


FIGURE V-4

The effect of Cyclosporin G (CsG), Cyclosporin A (CsA), and their respective metabolites on the release of endothelin by mesangial cells (A), and endothelial cells (B). The relative concentrations of metabolites in relation to parent drug are based on trough steady state levels. Results are expressed as mean $\pm$ SD ($n = 3$).



(potency ratio of 0.32) for cell growth in endothelial cells. The remaining metabolites had no appreciable effect on cell growth or DNA synthesis at the highest concentration tested of 25000 $\mu\text{g/L}$. Previous studies investigating the cytotoxic effects of CsA and its metabolites *in vitro* have shown that the latter in general are much less cytotoxic than parent drug (203,208-210). The IC_{50} for CsA was similar to that reported for CsG in the present study. This suggests that for both drugs, the relative activities of metabolites in relation to parent drug in inhibition of these cellular functions are similar.

The mechanism by which CsA induces chronic renal side-effects is not known. One hypothesis suggests that vasoconstriction of the arterioles and mesangium is responsible for the nephrotoxicity seen during drug therapy (47,48). This can be induced by the alteration in the production of prostanoids and other vasoactive substances. A balance between these substances is required to maintain a normal vascular tone; alterations in their production could lead to increased vasoconstriction. Two vasoactive substances were examined in this study: ET-1 a potent vasoconstrictor (211,212); and PGI_2 , a potent vasodilator (106,213).

In a preliminary set of experiments, the effect of individual CsG metabolites on release of PGI_2 and ET-1 was investigated. Two concentrations of drug and metabolite (500 and 5000 $\mu\text{g/L}$) were investigated. These latter concentrations are significantly higher than the respective trough concentrations found in blood of transplant recipients. However, the

intracellular rather than the extracellular concentration of the drug and metabolites may be more significant. We did not quantitate intracellular concentrations of metabolites in our study. However, it has been previously shown for CsA that tissue concentrations for both parent drug and metabolite are higher than those found in whole blood (57). A similar situation may exist for CsG. CsG, at both concentrations, did not have a statistically significant effect on the release of either ET-1 or PGI₂ as compared to control cultures in mesangial and endothelial cells. Only two metabolites were shown to have any effect on the release of vasoactive substances; GM1, at 5000 µg/L increased the release of ET-1, and GM9 at both 500 and 5000 µg/L, decreased the release of PGI₂ in mesangial cells. The remaining metabolites tested did not affect the release of the vasoactive substances tested from both cell lines. In view of the importance of mesangial and endothelial cells in the maintenance of vascular tone, these *in vitro* findings suggest that these CsG metabolites could have an effect on renal vascular tone *in vivo*. Previous studies with CsA have revealed a dose dependent reduction in PGI₂ release from mesangial cells over control values (203). A similar reduction was observed for most of the CsA metabolites on an individual basis. Conversely, the effect on the release of ET-1 was much less pronounced. The majority of metabolites did not effect the release of ET-1, but there was a significant increase in the production induced by metabolites AM19, AM1, and AM1c9 (203), suggesting these metabolites may lead to alteration in renal vascular tone.

To more appropriately compare the effects of CsA to CsG on the release of vasoactive substances *in vitro*, parent drug in combination with its metabolites at therapeutically relevant concentrations were investigated. The concentrations of parent drug chosen were 200 and 500 $\mu\text{g/L}$, since they span the therapeutic range for the drug (201,59). Metabolites were added to the medium at concentrations relative to that of parent drug observed at trough steady state measured in renal transplant recipients (57,167-169). Both CsA and CsG in combination with their respective metabolites had no effect on the release of ET-1 or PGI_2 as compared to control cultures by mesangial cells. Also, the release of PGI_2 by endothelial cells was unaltered by CsA, CsG and their metabolites. In contrast, both CsA, CsG, and their respective metabolites resulted in a statistical increase in the release of ET-1 by endothelial cells, as compared to control, subsequent to 24 hours of incubation. These *in vitro* findings suggest that at therapeutically relevant concentrations, both CsA and CsG in combination with their metabolites have a similar effect in stimulating release of ET-1.

In vivo studies have suggested a role for ET-1 as a mediator of renal ischemia in CsA induced renal vasoconstriction. The systemic infusion of high concentrations of CsA in animals has been shown to produce a marked elevation of ET-1 in plasma (214,215). In a rat model, Kon et al have demonstrated the alterations in renal function induced by CsA could be prevented by ET-1 blocking antibodies (214). In CsA treated renal, heart,

heart-lung, and liver transplant recipients, a significant increase in plasma ET-1 reaching a peak 6 h post-dose that followed the peak increase in CsA parent compound and which preceded the peak increase in metabolites has been reported (216). This data suggest that parent drug rather than metabolites is responsible for stimulating release of ET-1. However, direct measurement of metabolites was not performed. The metabolite concentrations were only estimated based on the difference in values obtained with specific and non-specific assays used for measurement of CsA. Such an approach can underestimate the concentrations of metabolites due to limited cross-reactivities of the antibodies employed for some metabolites (54).

The above results indicate that CsG and its metabolites have a potency comparable to CsA and metabolites on the release of ET-1 from endothelial cells. If the *in vitro* results hold for the *in vivo* situation, this suggests that the differences in renal side-effects between the two drugs do not reside in a differential effect of release of ET-1. The findings also suggest that the metabolites of both drugs may directly alter the renal neurodynamics, which may lead to the chronic renal side-effects *in vivo*.

VI. DISTRIBUTION OF MYCOPHENOLIC ACID IN BLOOD

1. RATIONALE:

To investigate the relationship between concentration of MPA and clinical efficacy, the most appropriate medium for measurement of the drug is required. However, this is not known since the distribution of the drug among elements in whole blood has not been determined. Such information would be useful to establish guidelines for monitoring of the drug and to facilitate centre to centre consistency in therapeutic evaluations. The distribution of MPA among whole blood components was therefore investigated.

2. MATERIALS AND METHODS

a. Source of drugs and specimens

MPA and RS-60461-0000 (E-6-[1,3-dihydro-4-(4-carboxybutoxy)-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl]-4-methyl-4-hexanoic acid) were obtained as gifts from Syntex Inc. (Palo Alto, Ca). Whole blood was collected from normal subjects by venipuncture into tubes containing EDTA as an anticoagulant. Specimens were pooled for each experiment. Plasma, obtained from Canadian Red Cross blood containing ACD as an anticoagulant, was stored frozen at -70°C prior to analysis.

b. Drug measurement

The amount of the drug was determined by HPLC as previously described

(217) with some modifications. Briefly, to 0.5 mL of sample, 1.5 mL of water, 100 μ L of a stock solution of internal standard RS-60461-000 (10000 μ g/L), and 0.5 mL of 0.15 M HCl were added. The sample was added to a pretreated Supelclean C-18 (Supelco Inc, Bellefonte, PA) solid phase extraction column, washed with 1.0 mL of water and eluted with 1.0 mL of 80% (v/v) MeOH in 0.1 M acetate buffer pH 4.0. Chromatographic separation of MPA and the internal standard was achieved with a C-18 Novopak HPLC column (Waters Inc., Milford MA) and a mobile phase of 27% (v/v) acetonitrile in 0.5% (v/v) phosphoric acid. The chromatographic eluate was monitored at 254 nm with the concentration of MPA being determined by the ratio of its peak height in relation to that of the internal standard. A standard curve was prepared using calibrators of MPA prepared in our laboratory. The assay has a sensitivity of 50 μ g/L and exhibits a linear range up to 10000 μ g/L. Concentrations above this were appropriately diluted with drug free plasma. Between-run coefficients of variation were <10% at 500 μ g/L and <7% at 1000 μ g/L.

c. Temperature equilibration studies

Whole blood was spiked with MPA at 500, 1000, 5000, and 10000 μ g/L since these concentrations span the ranges seen *in vivo* when immunosuppressive dosages are given (178,217). The blood specimens were incubated for a minimum of 2 h at 4° or 22°C to allow equilibration to occur. These specimens were then re-equilibrated for increasing lengths of time (0, 15, 30, 45, 60, 90, and 120 min) at 37°C prior to separation of cells by

centrifugation for 10 min at 2000 xg. The experiments were performed on three separate days ($n = 3$). Results were expressed as the ratio of the amount of MPA found in the plasma fraction versus the total added to whole blood (plasma/WB ratio).

d. Free fraction determinations

The free or unbound fraction of MPA was determined by ultracentrifugation as previously described (55,218,219). This method exhibits good correlation with such procedures as equilibrium dialysis and ultrafiltration (219). MPA was added to pooled human plasma to obtain the following concentrations: 100, 500, 1000, 5000, 10000 $\mu\text{g/L}$. The plasma samples were placed in 13 mL polyallomer ultracentrifuge tubes (Beckman Inc. Alto, Ca) and allowed to equilibrate. The tubes were placed in a T170 rotor followed by temperature-controlled (37°C) centrifugation (Beckman L8-70M) for 15 h at 150000 xg. The centrifuge was allowed to stop without braking to minimize turbulence. Aliquots (0.5 mL) were obtained from top to bottom of each tube. The fractions were split with a proportion being used for determination of protein content, the remainder being used for measurement of MPA. Fractions containing <0.5 g/L of protein were used to determine the amount of unbound MPA. The free fraction was determined as the total amount of drug in the fractions containing <0.5 g/L protein divided by the total amount of drug added.

e. Albumin binding

MPA was added to plasma as described above. Albumin was selectively removed from human plasma using the following procedure. Plasma samples were pretreated using gel-filtration columns (Bio-spin Chromatography Columns, Bio-Rad Laboratories Richmond Ca.) to remove small molecular weight compounds. The removal of albumin was achieved by applying the prepared sample to albumin binding affinity columns containing Cibacron-Blue F3GA (Econo-Pac Blue Dye affinity support, Bio-Rad Laboratories Richmond Ca.). This procedure has been shown not to displace non-covalently bound ligands from albumin (220). The cartridge was washed with 15 mL of buffer-I (20 mM Na_2HPO_4 , pH 7.1). The effluent contained the plasma proteins devoid of albumin. The bound albumin was then eluted from the cartridge by washing with 15 mL of buffer-II (1.4 M NaCl 20 mM Na_2HPO_4 , pH 7.1). The efficacy of the isolation procedure was confirmed by protein electrophoresis. The two fractions, one containing albumin and the second containing the other plasma proteins, were assayed for MPA as described above.

f. Lipoproteins binding

Very low density (VLDL), low density (LDL), and high density lipoproteins (HDL) were separated by ultracentrifugation as previously described (219). Briefly, the density of plasma was adjusted to 1.21 kg/L by the addition of potassium bromide (KBr). The plasma was supplemented with MPA as described above. To a 13 mL ultracentrifuge tube 2.5 mL of solution A (11.4

g/L NaCl, 0.33 g/L EDTA, 0.130 g/L sodium azide) was added followed by 3.0 mL of solution B (3.73 g KBr plus 200 mL of solution A) in order to float solution A above solution B. This was followed by 3.0 mL of solution C (16.68 g KBr plus 200 mL of solution A) and 4.0 mL of plasma. The samples were centrifuged for 24 h at 4°C at 200000 xg in an ultracentrifuge as described above. Following centrifugation the lipoprotein fractions, VLDL, LDL, HDL, and plasma residue (albumin and other major plasma proteins) were collected. The identity of each fraction was confirmed by lipoprotein electrophoresis (221). The amount of drug was measured in each fraction as described in section VI-2b and expressed as a percent of the total drug added.

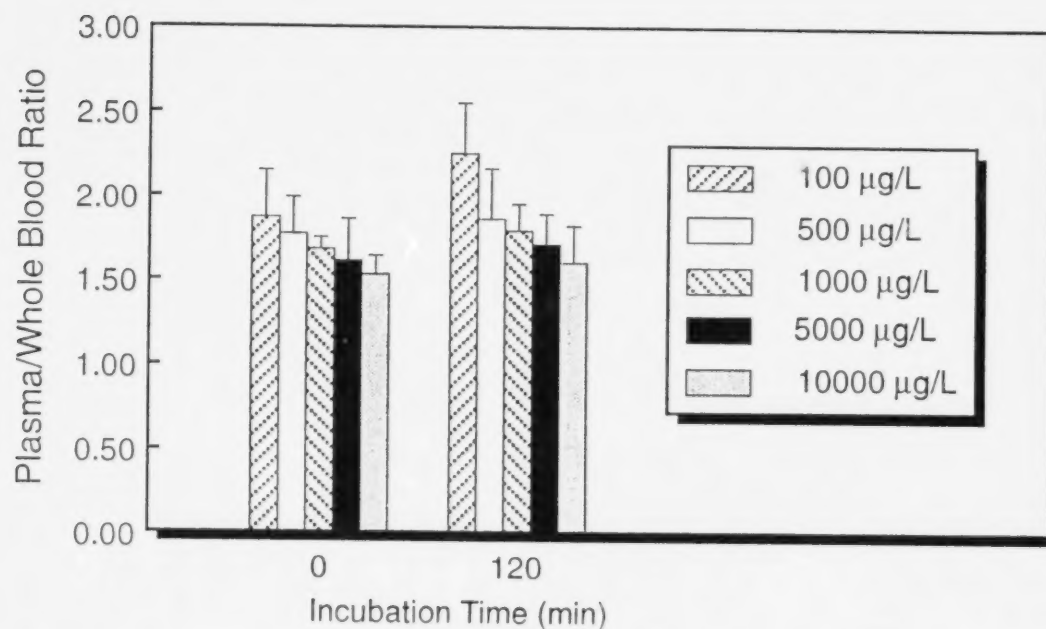
3. RESULTS

a. Temperature and equilibration studies

The plasma/whole blood ratio of MPA versus time of re-equilibration at 37°C for whole blood having an initial temperature of 4°C is shown in Figure VI-1. The distribution of MPA at the concentration range of 100-10000 µg/L did not change throughout the incubation intervals, and therefore does not exhibit a temperature dependence. Identical results were obtained for blood having an initial temperature of 22°C. The drug is found almost exclusively ($\approx 100\%$) in the plasma fraction. This results in a plasma/whole blood ratio of 1.5-2.0.

Figure VI-1

Effect of temperature on mycophenolic acid (MPA) distribution in whole blood.



The effect of Temperature on MPA distribution. Blood having an initial temperature of 4°C was re-equilibrated at 37°C for the times indicated prior to removal of cells. The whole blood concentration of MPA ranged from 100-10000 µg/L. Results are expressed as the ratio of MPA found in plasma to whole blood and represent the mean $\pm$ SD of duplicate experiments performed on 3 separate days (n = 6).

b. Free fraction

Subsequent to ultracentrifugation, 12 of the 0.5 mL (numbers 3-14) aliquots were found to be devoid of protein (ie. protein < 0.5 g/L). These aliquots were used to determine the free fraction of MPA. Figure VI-2 shows the majority of the drug is protein bound and exhibits a concentration dependency with a proportional increase in the free fraction as total drug concentration increases. The percentage of MPA which is free or unbound ranges from 7.3-16.8% of total drug for concentrations ranging between 500-10000 $\mu\text{g/L}$.

c. Albumin binding

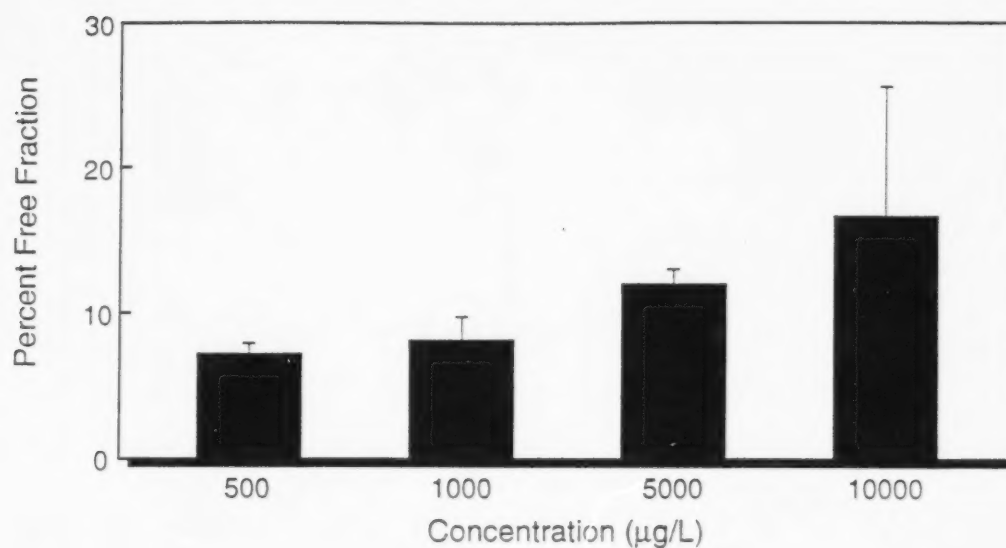
Subsequent to the separation of albumin from other plasma proteins the concentrations of MPA was determined in each. The majority of the drug is bound to non-albumin proteins (44-69%), with a lesser amount bound to albumin (approximately 8-30%) (Table VI-1). As the concentration of MPA increases from 1000-10000 $\mu\text{g/L}$ the proportion of drug bound to albumin decreased with a corresponding increase in the remaining protein fraction.

d. Lipoprotein binding

To further investigate the binding of MPA to proteins in the non albumin fraction in plasma, the binding of the drug to chylomicrons, VLDL, LDL, and HDL was investigated for concentrations ranging from 500-10000 $\mu\text{g/L}$. The results are shown in Figure VI-3. Of all lipoprotein fractions, HDL bound the largest proportion of MPA (3-8%), with a lesser amount bound to LDL (0.5-

FIGURE VI-2

Free fraction of mycophenolic acid (MPA) as determined by ultracentrifugation.



Free fraction of MPA as determined by ultracentrifugation. Initial plasma concentrations of MPA are listed. Results represent the mean $\pm$ SD of duplicate experiments performed on 3 separate days ($n = 6$).

Table VI-1

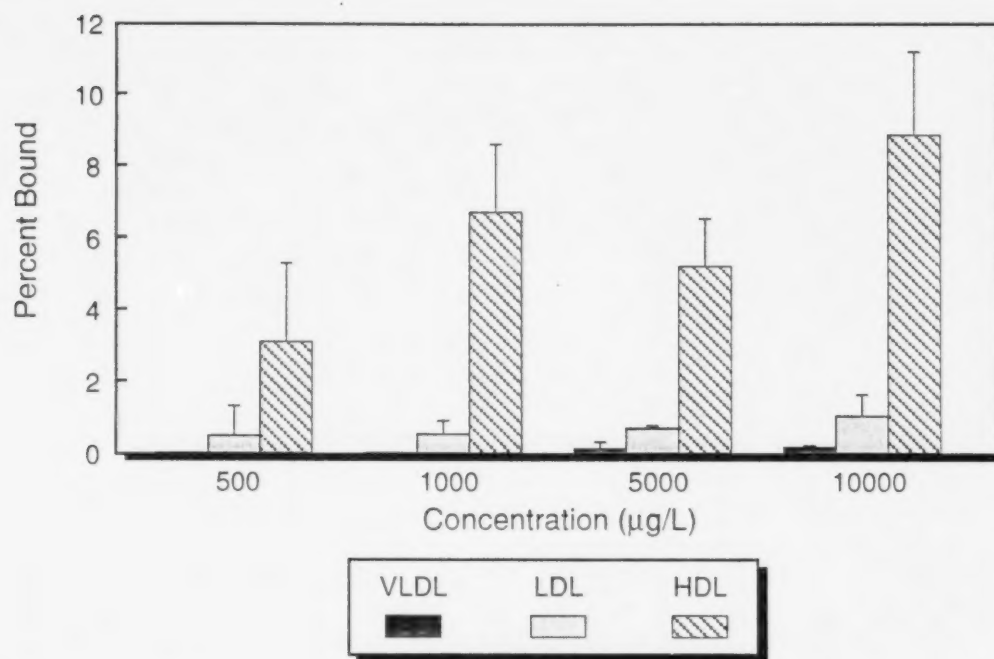
Binding of mycophenolic acid (MPA) to albumin

Concentration of MPA ($\mu\text{g/L}$)	mean (% associated with albumin)	SD
500	N/D ¹	-
1000	30.1	5.2
5000	19.3	1.7
10000	7.5	1.2
25000	6.6	2.9

¹ result was less than sensitivity of the assay.

FIGURE VI-3

Binding of mycophenolic acid (MPA) to lipoproteins.



Binding of MPA to lipoproteins. MPA was added to plasma at concentrations from 500 - 10000 µg/L and was followed by isolation of the lipoproteins by ultracentrifugation. Results are expressed as the mean $\pm$ SD of duplicate experiments performed on 3 separate days ($n = 6$).

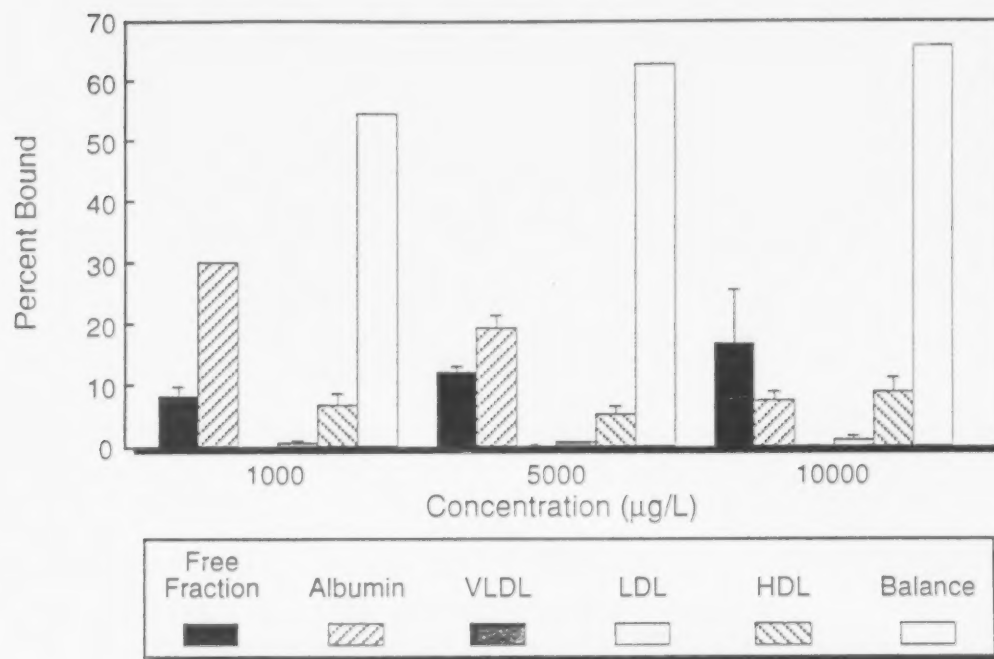
1.9%) and VLDL (<0.2%). The binding to lipoproteins did not exhibit a concentration dependence.

4. DISCUSSION

The concentration ranges that were chosen in this study were from 500-10000 $\mu\text{g/L}$, which represent those that were attained during therapy when immunosuppressive doses were given (178,217). Virtually all the drug is found in the plasma fraction. Plasma/whole blood ratios of 1.5-2.0 were obtained due to the dilution factor of erythrocytes in the latter fraction. The overall distribution of MPA in plasma from the studies described is represented in Figure VI-4. It can be concluded that, in plasma, MPA exhibits concentration independent binding to lipoproteins (<10%). It should be noted that the high salt concentrations used in the isolation of lipoproteins may have resulted in some dissociation of MPA from lipoproteins. However, this procedure has been the most commonly used to investigate the binding of immunosuppressive drugs to lipoproteins (222-224). These studies would also be subject to the same difficulties. The drug is bound to albumin in a concentration dependent manner that appears to be saturable. The concentration of MPA that is required to maximally occupy albumin binding sites is approximately 5000 $\mu\text{g/L}$. Consequently, the free fraction would increase as the concentration of MPA increases, this is supported by our findings that the free fraction exhibits a dose dependent relationship. The converse is true for the free fraction. It is

FIGURE VI-4

Overall distribution of mycophenolic acid (MPA) among plasma components.

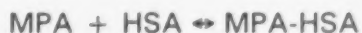


Overall distribution of MPA among plasma components. Data was obtained as described in methods and summarizes the results in Figures VI-1 to VI-3 above. Results are expressed as the mean $\pm$ SD of duplicate experiments performed on 3 separate days ($n = 6$).

generally accepted that the free or unbound drug is the most pharmacologically active moiety (221). This infers that as total MPA concentration increases, the biological effect of the drug would increase in a greater proportion than the concentration of MPA until drug binding sites or proteins become saturated. In the case of albumin, a substantial amount of MPA is bound to plasma proteins other than albumin and lipoproteins. At present, the identity of these proteins has not been determined.

The above findings are in contrast to what other researchers have found, using an ultrafiltration method for the measurement of unbound MPA (179). It was shown that MPA is extensively bound to human serum albumin (HSA), which is constant over a broad concentration range (179). However, the binding of MPA to HSA was performed in the absence of other plasma proteins, while in the current study an attempt was made to remove the albumin from the plasma matrix. This may account for the difference in the two studies.

Another limitation in evaluating the MPA binding to albumin using the column separation method involves the equilibrium reaction between the free fraction of MPA and binding to albumin.



If the MPA-HSA complex is easily dissociated, then use of Cibacron-Blue F3GA columns may not be the most appropriate method for the separation of HSA bound to MPA. However, if the rate of dissociation of MPA from HSA is slow, then the columns could be used for purification of the MPA-HSA complex.

Most drugs are reversibly bound to plasma proteins, the avidity and extent of the binding varying with the drug. Most acidic, neutral, and basic drugs bind almost exclusively to HSA, although a few basic drugs bind to α_1 -acid glycoprotein (225). The degree of binding of a drug to plasma proteins and tissues can govern its elimination from the body, and ultimately, its therapeutic or toxic effect. It is generally believed that only the unbound form of a drug interacts with its receptor to produce effects. This principle is the basis for the understanding of PK behaviour and the pharmacological activity of the drugs that are avidly and extensively bound to plasma proteins at therapeutic concentrations (225).

It was reported that protein binding significantly affected the interaction of MPA with purified IMPDH, and the interaction of MPA with peripheral blood mononuclear cells (179). The IC_{50} for IMPDH type II increased 5.4 fold as the concentration of HSA increased from 0 to 50 g/L. Thus, as the degree of MPA binding to HSA increased and the free fraction decreased, there was a corresponding decrease in the inhibition of IMPDH activity for a given drug total (179). Furthermore, the addition of 10 g/L HSA to culture medium containing PHA-stimulated peripheral blood mononuclear cells increased the IC_{50} of MPA by 4.8 fold. Therefore, the pharmacologic effect was more directly related to the free rather than the total drug concentration (179).

Many reports describe reduced drug binding in diseases including; liver disease, various types of renal dysfunction, burn injuries, and malnutrition

(179,226). Concurrently administered drugs can also alter the binding of MPA. Many commonly used drugs including CsA, FK-506, prednisone, coumadin, heparin, and acetaminophen have no effect on MPA binding, while sodium salicylate and ethanol decrease the binding of MPA leading to an increased free fraction (179). It is the free fraction that is available for metabolism, and clearance. The clearance of drugs may increase in proportion to an increase in the free fraction (225). Thus, transplant patients with low albumin concentrations could have reduced binding of MPA and potentially an increase in clearance. It was also found that uraemia will cause decreased binding of many drugs (226). Therefore, it is predictable that renal transplant patients in the early post-transplant period could have altered MPA binding (177). Altered binding of MPA during this period may have an impact on the PK, potentially requiring altered dosing regimes and monitoring strategies during this time.

The distribution of MPA is different from other immunosuppressive drugs like Cyclosporin A (CsA), Cyclosporin G (CsG), FK506 and rapamycin (RAPA) (55,218,227,228). CsA, CsG, and FK506 exhibit temperature dependent distribution; increasing temperatures results in a proportional increase in the concentration of the drug in plasma (55,227,228). In whole blood, CsA, CsG and FK506 are primarily bound (>50% of total drug concentration) to erythrocytes (227,228). With RAPA, greater than 95% of the total drug is bound to this fraction; however, the distribution does not show any temperature dependence (218). Based on their distribution, whole blood is the

medium of choice for the measurement of these four drugs (55,218,227,228).

This study, in contrast, concludes that from an analytical standpoint plasma is the most suitable medium for analysis of MPA, due to the higher concentrations found in this fraction.

VII. DEVELOPMENT OF AN ASSAY FOR INOSINE MONOPHOSPHATE DEHYDROGENASE (IMPDH)

1. RATIONALE:

Therapeutic monitoring of immunosuppressive drugs has traditionally involved the measurement of drug concentrations in whole blood or plasma. However, it may be difficult to assess the therapeutic concentrations of individual drugs in patients receiving multi-immunosuppressive drug therapy. An alternative approach involves the measurement of the effect of the drug, not its concentration, to assess the state of immunosuppression. As MPA inhibits the enzyme IMPDH, the pharmacodynamic monitoring of the degree of enzyme inhibition may provide a better indicator of immunosuppression as compared to drug concentrations in plasma. We report here on the establishment of an assay for the measurement of IMPDH in whole blood and the relationship between MPA concentrations and the degree of inhibition of this enzyme.

2. MATERIALS AND METHODS

a. Source of drugs

MMF, MPA and the glucuronide metabolite of MPA (MPAG) were obtained as gifts from Syntex Inc. (Palo Alto, Ca). Whole blood was collected from normal human volunteers, male New Zealand White rabbits, and mongrel

dogs into tubes containing heparin, EDTA, or ACD as an anticoagulant. The procedures followed were in accordance with the ethical standards of the University of Alberta.

b. Isolation of lymphocytes

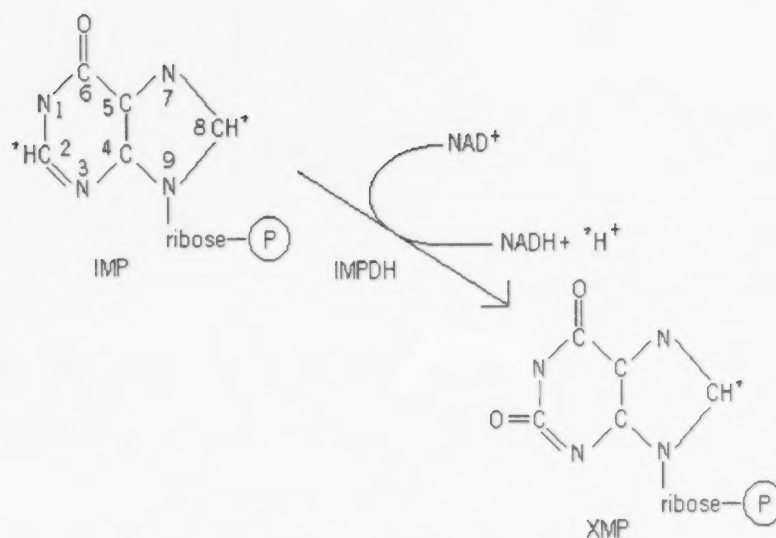
Separation of lymphocytes was performed as previously described (55,218). Pooled fresh whole blood was layered over an equal amount of Histopaque 1077 (Sigma Diagnostics Ltd. St Louis MO) and centrifuged for 30 min at 400 xg. The resulting plasma and mononuclear layer were removed and washed twice with RPMI medium (Gibco Laboratories, Burlington, ON, Canada). The platelets and monocytes were removed from the suspension by adding a drop of Thrombostat 5000 IU/L (Parke-Davis, Scarborough, ON), followed by centrifugation for 60 sec at 400 xg. The purity of the lymphocytes in the supernatant as determined microscopically was found to be > 95%. For experiments other than those specified, the isolated lymphocytes were incubated for 30 min in RPMI prior to determination of activity as described below.

c. Determination of IMPDH activity in isolated lymphocytes

The activity of IMPDH in intact lymphocytes was measured using a modification of a procedure described by Balzarini and De Clercq (229). Briefly, the enzyme activity was determined by the estimation of the ^3H released (Figure VII-1) from [2,8- ^3H] IMP that had been formed in the cells from [2,8- ^3H]hypoxanthine (Hx) (15 Ci/mmol) or [2,8- ^3H]inosine (Ino) (30 Ci/mmol)

Figure VIII-1

Diagrammatic representation of IMPDH assay

adapted from Lehninger AL. Principals of Biochemistry 1982.

(Moravek Pharmaceuticals, Brea CA, USA). During the reaction, the tritium atom located on C-2 of the hypoxanthine ring of IMP is replaced by a hydroxy group. NAD^+ serves as the electron acceptor, and is reduced to NADH. The rate of catalysis with the ^3H substrate is less than that of non-labelled substrate due to the tritium isotope effect (230). The procedure involved the use of 500 μL of lymphocytes at a concentration of 2.5×10^6 cells/L, to which was added 100 μL (10 μCi) of radio-labelled Hx or Ino. At time points 0, 10, 20, and 30 min 100 μL aliquots were removed and mixed with 500 μL of freshly prepared cold suspension of 100 mg/ml activated charcoal in 5% (w/v) trichloroacetic acid. Subsequent to centrifugation at 1300 $\times g$ for 10 min, 200 μL of the supernatant was analyzed for radioactivity by scintillation counting (Minaxi β Tri-carb 4000 series, Canberra Packard, Canada) using a quench corrected counting program. The amount of spontaneous liberation of ^3H was measured with each analysis and was determined to be $< 1.0\%$ of the amount of Hx or Ino added. The amount did not change significantly with time and this background was subtracted from all values to obtain net values for the amount of ^3H released enzymatically by IMPDH. The enzyme activity was determined from the slope of the graph of ^3H release versus time and was expressed in disintegrations per minute per minute (dpm/min).

d. Determination of IMPDH activity in whole blood

The determination of IMPDH activity in whole blood was achieved by using 500 μL of whole blood in place of the lymphocytes in the procedure

described above. For experiments other than those specified, MPA was added to blood 30 min prior to analysis.

e. Effect of WBC concentration on IMPDH activity

To determine the effect WBC concentration in whole blood may have on total IMPDH activity, whole blood from normal individuals was layered over an equal amount of polymorphoprep (Nycomed Pharma, Oslo, Norway). Subsequent to centrifugation for 30 min at 600 xg the two layers of WBC (polymorphonuclear and mononuclear cells) were removed and washed twice with saline. The RBC layer devoid of WBC was washed twice with saline and resuspended in plasma. This represented the zero WBC standard for whole blood (WB). Plasma was added to the isolated WBC, and the cells were resuspended by gentle mixing. The cell suspension was used to reintroduce WBC at concentrations of 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 16.0x10⁹ cells/L. Similar experiments were performed for WBC resuspended in both saline and RPMI in place of WB. The WBC determination was performed using an cell automated counter (Coulter STKS, Hialeah, FL). The enzyme activity was subsequently determined in the samples. Independent experiments were performed on three separate days (n = 6).

f. Statistics

Unless specified, all experiments were performed in duplicate on a minimum of three separate occasions (n = 6). Results are expressed as mean $\pm$ SE. Statistical differences between samples were tested using a two-way

analysis of variance or paired t-tests using SPSS statistical software (SPSS Inc. Chicago, IL).

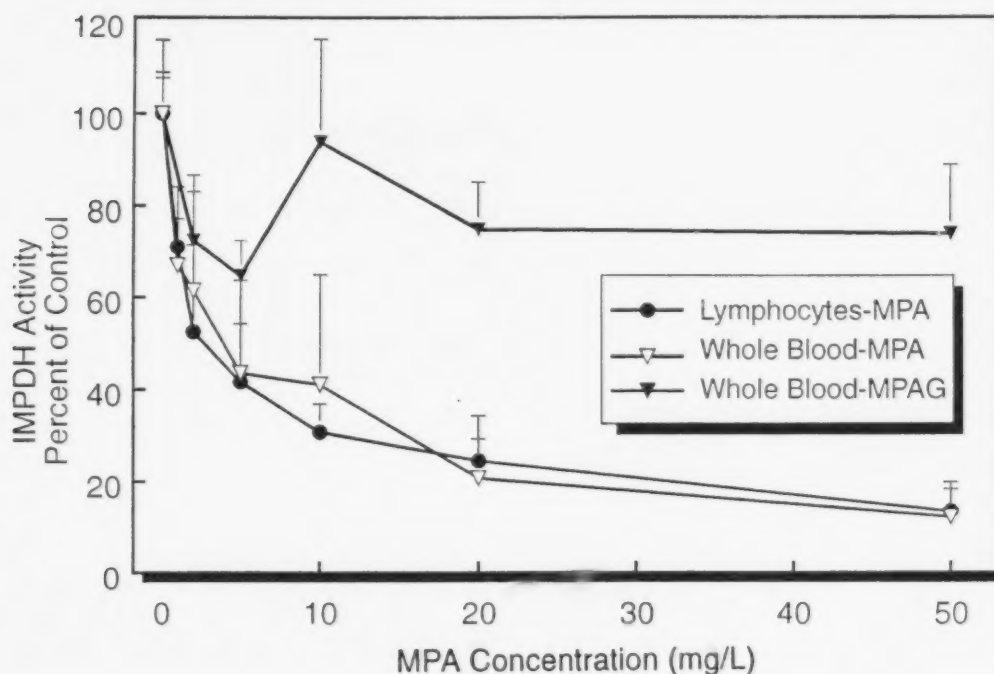
3. RESULTS

a. IC_{50} studies

MPA was added up to a concentration of 50.0 mg/L to whole blood and human lymphocytes suspended in tissue culture medium. The vehicle, which consisted of methanol/Tween 80 70:30 (v/v) did not affect IMPDH activity as the final methanol concentration was $<0.2\%$ (v/v). As shown in Figure VII-2, the concentration of MPA required for 50% inhibition of IMPDH activity was similar for both whole blood and isolated lymphocytes, in the range of 2.0-5.0 mg/L. However, the concentration required for 50% inhibition of enzyme activity induced by the glucuronide metabolite of MPA, MPAG, was greater than the concentrations tested; at 100 mg/L MPAG only 74% inhibition was observed. Similar results were obtained for rabbit whole blood. The *in vivo* IC_{50} for dogs is shown in Figure VII-3. As the concentration of MPA increased there was a proportional decrease in IMPDH activity. At a concentration of 200 mg/L the inhibition of IMPDH activity was $58 \pm 8.3\%$ for whole blood ($n=6$) and $55 \pm 10.0\%$ for isolated lymphocytes ($n=6$). There was no statistical difference in the degree of inhibition of IMPDH activity by MPA at any of the concentrations tested in isolated lymphocytes or whole blood. Rabbits and humans have similar IC_{50} values while dogs have an IC_{50} approximately 100

Figure VII-2.

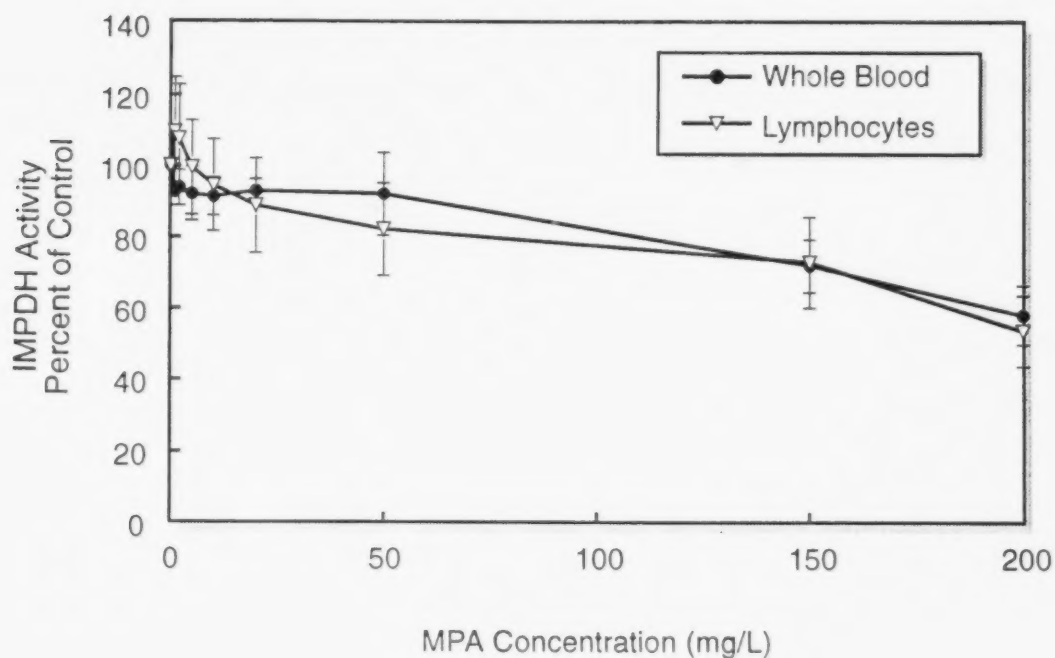
The effect of mycophenolic acid (MPA) and mycophenolic acid glucuronide (MPAG) concentration on IMPDH activity



The effect of MPA and MPAG on IMPDH activity. Enzyme activity in whole blood and isolated lymphocytes from humans was expressed as % of enzyme activity in the absence of drug. Experiments were performed in duplicate on three separate days and the results are expressed as mean $\pm$ SE ($n=6$). The IMPDH activity in the absence of drug were 177.6 ± 33.4 dpm/min.

FIGURE VII-3.

The effect of mycophenolic acid (MPA) concentration on IMPDH activity in canine whole blood.



The effect of MPA concentration on IMPDH activity in canine whole blood. Enzyme activity in whole blood and isolated lymphocytes from dogs was expressed as % of enzyme activity in the absence of drug. Experiments were performed in duplicate on three separate days and the results are expressed as mean $\pm$ SE ($n=6$). The IMPDH activity in the absence of drug was 296.6 ± 71.2 dpm/min.

fold higher.

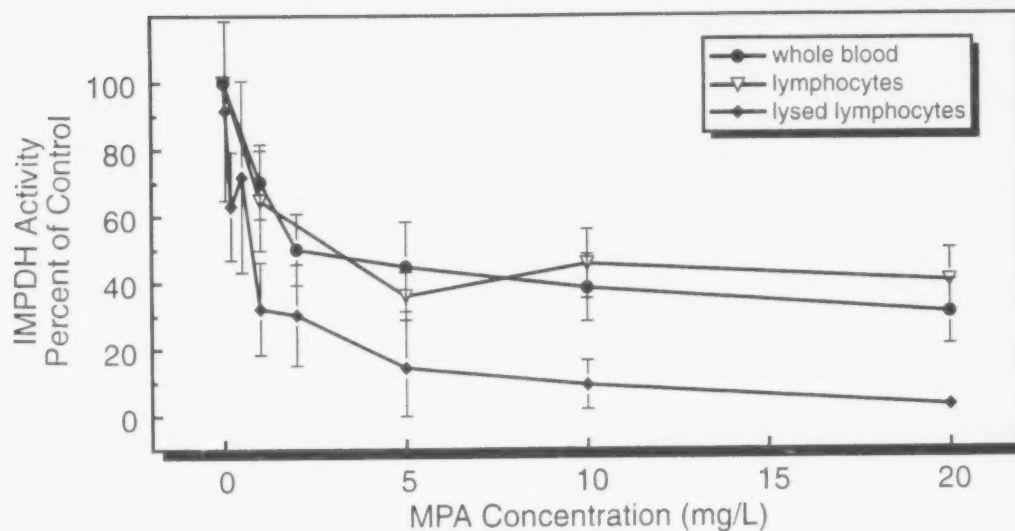
In human whole blood complete inhibition of IMPDH was not observed at the highest concentration of MPA investigated. However, when cell membranes were disrupted in the presence of 0.1% Triton-X-100 (Sigma Diagnostics, St.Louis, MO) which alone did not inhibit enzyme activity, the IC_{50} was found to be approximately 0.5 to 1.0 mg/L (Figure VII-4). Complete inhibition of enzyme activity was achieved at a MPA concentration of approximately 20 mg/L. This suggests that any further metabolism of xanthine monophosphate or excess inosine monophosphate to uric acid, which would result in the release of ^3H from the C-8 position is insignificant. In a separate experiment, lymphocytes were isolated from whole blood containing MPA at a concentration of 10.0 mg/L followed by measurement of IMPDH activity. No inhibition of IMPDH activity was observed, suggesting a reversal of MPA induced inhibition during the isolation of lymphocytes due to loss of MPA from the cells upon isolation. For this reason the remainder of the analysis was performed using whole blood as the medium for analysis.

b. Effect of WBC count on IMPDH activity

Whole blood devoid of WBC was added to the latter to attain concentrations ranging from 0.0-16.0x10⁹ cells/L followed by measurement of enzyme activity. The results shown in Figure VII-5, indicate a relationship between WBC and IMPDH activity. As the WBC numbers increase a proportional increase in IMPDH activity was observed. The graph appears linear

FIGURE VII-4

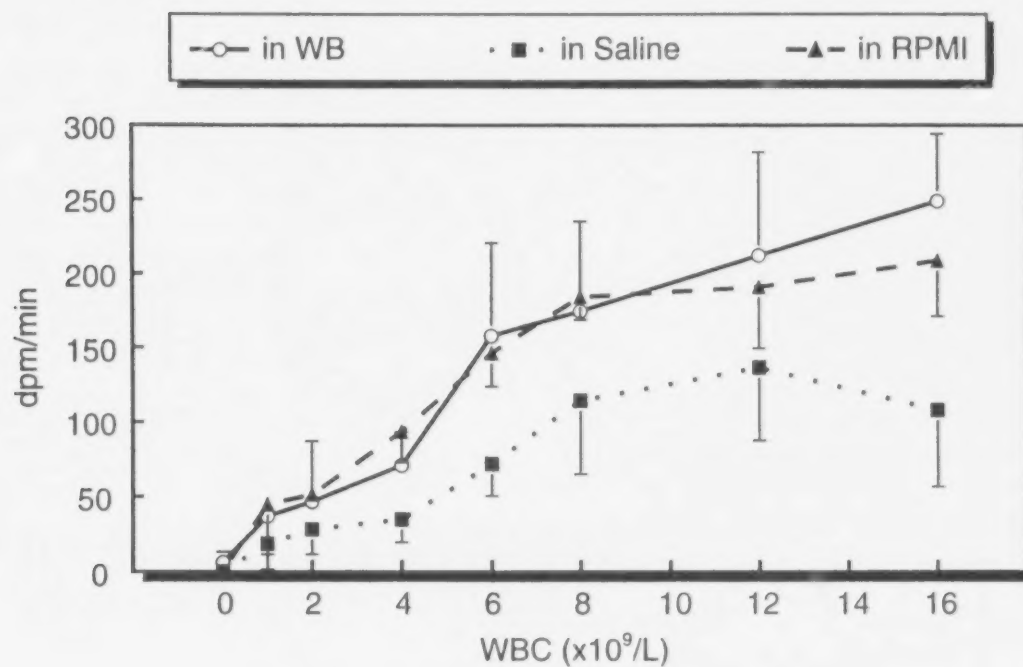
The effect of mycophenolic acid (MPA) concentration on IMPDH activity.



The effect of MPA on IMPDH activity. Enzyme activity in human whole blood, isolated lymphocytes, and lymphocytes treated with Triton X-100 was expressed as % of enzyme activity in the absence of drug. Experiments were performed in duplicate on three separate days and the results are expressed as mean $\pm$ SE ($n=6$). The IMPDH activity in the absence of drug was 148.6 ± 43.2 dpm/min.

FIGURE VII-5

The effect of white blood cell (WBC) concentrations on IMPDH activity.



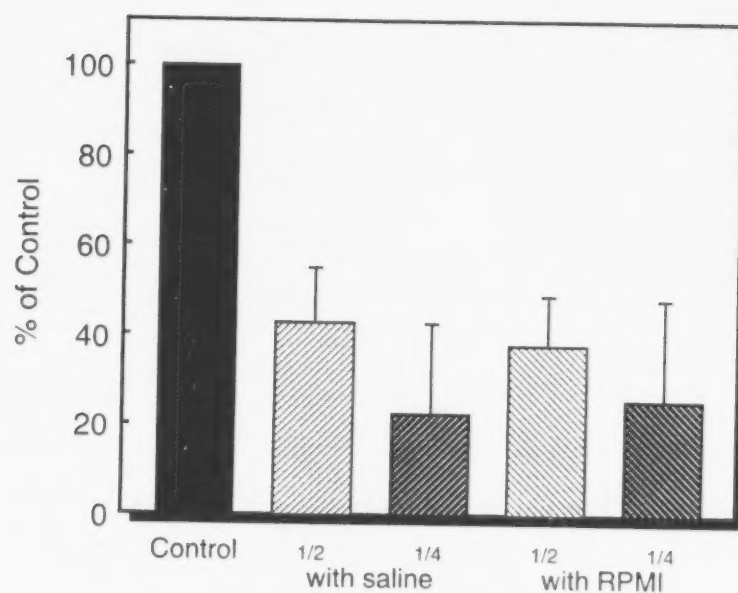
The effect of WBC on IMPDH activity. Experiments were performed in duplicate on three separate days and the results expressed as the mean $\pm$ SE ($n = 6$).

within what is generally accepted as the normal range for WBC counts ($4.0-9.0 \times 10^9/L$). This suggests that a correction for the WBC count can be made within this range. There is no statistical difference between the values at the various WBC concentration in WB, RPMI, or saline. The WBC suspended in saline however had a trend to lower IMPDH activity, which was not significantly different. Isolated fractions of platelet rich plasma and plasma alone had IMPDH activities of $<1.0\%$ of the total IMPDH activity. The red blood cell (RBC) fraction had an IMPDH activity of $<10.0\%$ of total, however, when a WBC count was performed on this sample it was apparent that approximately 80% of the total WBC population was removed. Therefore, the detectable IMPDH activity could be due, in part, to the remaining WBC. This suggests that for intra and inter-patient comparisons results could be normalized for WBC concentration if the patients WBC count falls within the established normal range. All values unless otherwise specified were normalized to a WBC concentration of 1.0×10^9 cells/L.

To determine if patient specimens having elevated WBC could be diluted to bring values within a lower concentration range to avoid substrate depletion, serial dilutions were performed. WB from normal volunteers was collected and diluted 1/2 and 1/4 with saline and RPMI. The IMPDH activity was measured and the results shown in Figure VII-6. The 1/2 dilution was approximately 40-50% of control, this was slightly less than the expected values but within the assay precision (see below). The 1/4 dilution, however, was approximately

Figure VII-6

The effect of dilution on IMPDH activity



The effect of dilution on IMPDH activity. Whole Blood was diluted 1/2 and 1/4 with Saline or RPMI. Experiments were performed in duplicate on three separate days and the results expressed as the mean $\pm$ SD ($n=6$). The IMPDH activity of the un-diluted sample was 212.8 ± 42.6 dpm/min.

25% of control values, but, the variation was much greater, as indicated by the large SE. This could be a reflection of the low WBC in the samples, since the IMPDH activities measured in these samples were near the low end of the assay range. These results suggest that patient specimens with elevated WBC counts could be diluted to bring values within the linear range. However, in the studies performed the specimens were corrected for the WBC subsequent to measurement of IMPDH activity. However, it may be more appropriate to dilute the samples to an predetermined standard WBC prior to analysis.

c. Assay precision

The intra- and inter-assay precision of the method for determination of IMPDH activity in whole blood from a single individual as well as the inter-individual variation from blood specimens (both normalized and not normalized for WBC) obtained from a number of healthy volunteers is shown in Table VII-1. The within and between run coefficients of variation for blood specimens obtained from a single individual were 11 and 16% respectively. The inter-individual variation in IMPDH activity in whole blood was not reduced when results were corrected for WBC in either human or rabbit whole blood indicating the large variability in the normal population (Table VII-1). However, the analysis was performed on normal subjects who exhibited minimal inter-individual variation of WBC count ($4.5-7.2 \times 10^9$ cell/L), which is in contrast to transplant patients, where a large variation may occur.

Table VII-1

Precision and inter-individual variation of IMPDH activity in whole blood^a

Precision

	intra-assay	inter-assay
N	6	7
mean	47.8 dpm/min	81.6 dpm/min
SD	5.16 dpm/min	13.2 dpm/min
CV	11%	16%

Inter-individual Variation^b

	n	non-normalized (dpm/min)	normalized ^c (dpm/min)
human	11	366 ± 148 (182 - 668) ^d	57.5 ± 26.2 (37.2 - 96.8)
	CV	40.4	45.5
rabbit	11	497 ± 120 (284 - 656)	61.6 ± 22.1 (26.6 - 105.4)
	CV	24.1	35.8

a -all results are corrected for background counts

b -results expressed as mean ± SD

c -results normalized for WBC to 1x10⁹/L

d -range of values

The effect of three common anticoagulants, lithium heparin, EDTA, and ACD, on IMPDH activity in whole blood was evaluated. Whole blood samples were collected and stored up to 7 days followed by the determination of IMPDH activity. The results indicated there was no statistical difference in IMPDH activity ($p < 0.05$) between any of the anticoagulants up to 1 week post sample collection when stored at 4°C (Figure VII-7). For storage times greater than this, a trend to decreased activity was noted.

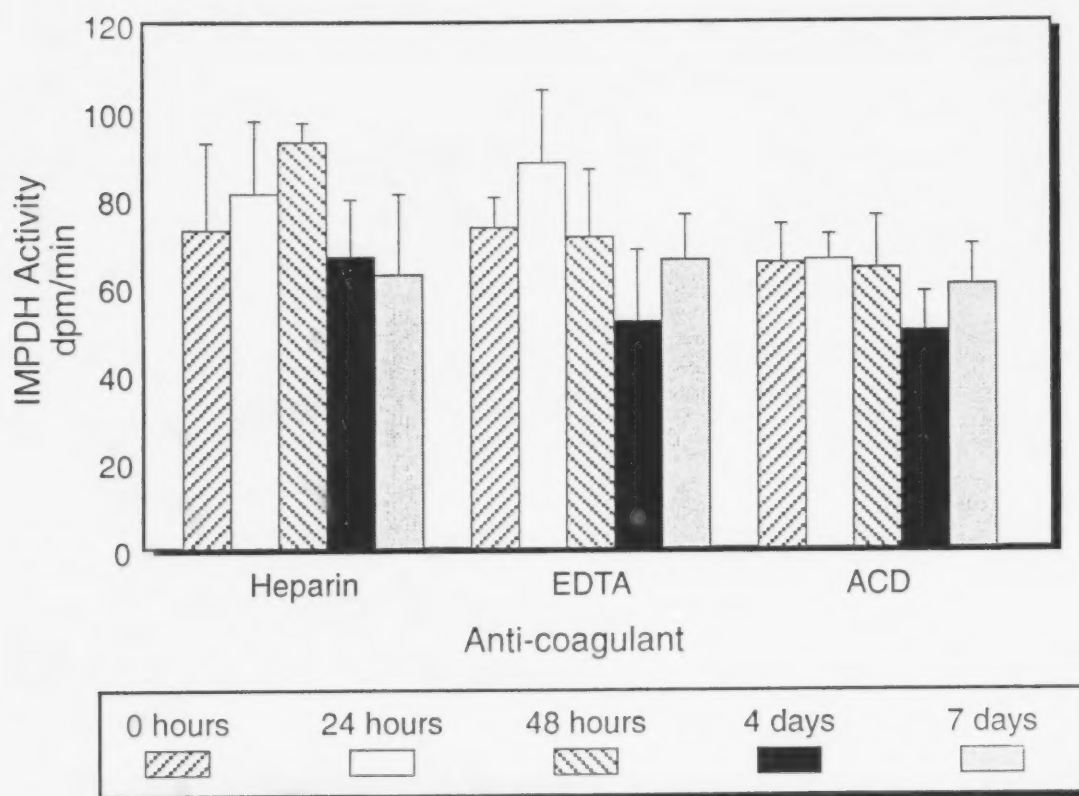
The effect of incubation time with MPA on IMPDH activity in whole blood was determined. MPA was added to WB to concentrations of 1.0 and 10.0 mg/L followed by incubation for up to 120 min prior to assay for enzyme activity. The inhibition of IMPDH by MPA was immediate and no change in activity occurred during the time studied (Figure VII-7).

4. DISCUSSION

The first phase of the work involved the establishment of a reproducible assay for the monitoring of IMPDH activity in whole blood since this medium would result in minimal sample preparation. The assay is based on the release of ^3H from IMP, the substrate of IMPDH (229). IMPDH exists in two isoforms type I and type II (41). The assay established for IMPDH as described here most likely measures the activity of both isoforms; however, this needs to be confirmed. The method is relatively simple to perform with up to 12 specimens being processed at the same time. The concentration required for 50%

FIGURE VII-7

The effect of anticoagulants and storage time on IMPDH activity



The effect of anticoagulants and storage time on IMPDH activity. Experiments were performed in duplicate on three separate days and the results expressed as the mean $\pm$ SE ($n=6$).

inhibition of IMPDH was approximately 2.0-5.0 mg/L for both whole blood and isolated lymphocytes. The limit of lowest concentration of MPA to give a statistically significant change in IMPDH activity is 1.0 mg/L. At concentrations above 20 mg/L no substantial increase in the inhibition of enzyme activity was observed, suggesting an effective range of 1.0-20 mg/L. This concentration range span those found in plasma when immunosuppressive doses of the drug are administered (178,217). MPAG, the glucuronide metabolite of MPA, had an IC_{50} of > 100 mg/L which indicates its inhibitory effect on IMPDH activity is $< 5\%$ of MPA. It has been shown that in renal transplant recipients the concentration of MPAG can be 100 fold greater than the parent MPA. Although MPAG has minimal effects *in vitro*, at these high concentrations in plasma the MPAG could contribute to the overall immunosuppression. This needs to be investigated

IMPDH activity was not inhibited in lymphocytes isolated from whole blood containing MPA *in vitro*. During the isolation procedure, MPA is lost from cells, resulting in the restoration of enzyme activity, indicating the inhibition of IMPDH by MPA is reversible. The inhibition of enzyme activity by MPA is almost immediate when drug is added to whole blood, thus indicating a rapid uptake of the drug into cells. However, complete inhibition of IMPDH activity was not achieved even at high concentrations of MPA, except when cell membranes were solubilized in the presence of a non-ionic detergent. There are several explanations for such observations. It is possible that MPA transport

is saturable, and MPA concentrations intracellularly are not sufficient to result in the complete inhibition of IMPDH. These observations could also be explained by the presence of an intracellular binding protein or receptor. There is the possibility that intracellular metabolism would reduce the amount of available MPA. There is also the possibility of diffusion either within the intracellular components or to the extracellular matrix thus reducing the effective concentration of the drug.

The evaluation of the relationship between IMPDH and MPA levels *in vitro* for canine blood and lymphocytes suggest that the concentration needed to inhibit IMPDH activity is approximately 200 mg/L. This is much greater than the IC_{50} in both rabbit and human whole blood. This suggests differences in uptake or responsiveness of canine IMPDH to MPA. In dogs, *in vitro*, much larger amounts of MPA are required in order to achieve the same pharmacological effect, implying that much greater dosage of the drug would be required as compared to humans and rabbits to achieve the same PD effect. However, at the doses used in these studies, adequate immunosuppression has been reported in some transplant models but not in others (82,231). Clearly the discrepant results in the dog model require further investigation.

The assay precision is similar to that of other manual methods that are used in clinical laboratories. Although some variation in enzyme activity was noted between patients, this was not substantially reduced when specimens were corrected for WBC. However, this was evaluated in a normal population.

The enzyme activity was not affected by the type of anticoagulant used and was stable in blood specimens stored for up to 48 hours at 4°C. This indicates that immediate analysis of the specimens is not required and is therefore more adaptable for routine clinical monitoring.

VIII. VALIDATION OF PHARMACODYNAMIC MONITORING OF MYCOPHENOLATE MOFETIL IN ANIMALS

1. RATIONALE:

The canine model has been used frequently to evaluate new immunosuppressive drugs. Experience has shown that dogs exhibit a greater sensitivity to immunosuppressive drug induced side effects as compared to other animal species (82,231). This has resulted in a trend to lower dosages to prevent toxic side effects. However, as detailed in the previous chapter, *in vitro* dogs appear to require greater amounts of MMF to inhibit IMPDH activity (the IC_{50} is approximately 100 fold greater than for rabbits or humans). The *in vivo* relationship between inhibition of IMPDH activity and MPA levels in this model was investigated to ascertain which dose or plasma concentration range results in optimal inhibition of the former.

In vitro rabbits exhibit similar responses to MPA as seen for humans. Similar studies to those described above for the dog were performed in the rabbit to determine the relationship between the PK and the PD effect of MMF. This information should facilitate the optimization of dosing strategies for MMF. If pharmacodynamic monitoring is to be used as a clinical method for dosage adjustment and optimization, its relationship to graft survival in a transplant model must be investigated. This was investigated in a rabbit heterotopic heart transplant rabbit model.

2. MATERIALS AND METHODS

a. Source of drugs and specimens for pharmacokinetic/pharmacodynamic studies

MMF was obtained as a gift from Syntex Inc. (Palo Alto, Ca). Whole blood (3 ml) was collected from both male and female normal mongrel dogs (weights ranging from 19.3-29.6 kg) into tubes containing EDTA as an anticoagulant. Following an overnight fast, the dogs were placed in a Pavlov restraining device. The hind leg saphenous vein was cannulated with a 16-G Medicath (Becton & Dickenson, Sandy Utah, USA), and a single dose of MMF at 20 or 40 mg/kg was administered orally, with at least 7 days recovery period between experiments. Blood samples were collected at the following times: pre-dose, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h post dose. The dogs were released from the restraint, the IV cannula removed, and the dogs were allowed food 4 h after the initiation of drug administration. Subsequent venous samples were drawn by venipuncture of the foreleg cephalic vein.

Whole blood (3 mL) was collected from New Zealand White rabbits who received MMF into tubes containing EDTA as an anticoagulant. Blood sample were collected via the marginal ear vein. Acute administration was evaluated in rabbits ($n=4$) who received a single dose of 40 mg/kg IV. Blood samples were collected at the following times: pre-dose, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h post-dose. Chronic administration was evaluated ($n=4$) at a dose of 80 mg/kg/d SC for 7 days. Blood samples were collected at the times described

above on day 0, the first day of administration, and on day 7.

The samples for IMPDH activity were stored at 4°C until the time of analysis. For determination of MPA concentrations, plasma was separated and stored at -20°C until analysis.

b. Surgical procedure

Heterotopic cardiac transplants were performed using male New Zealand White rabbits as recipients and donors (2.5-3.0 kg). The recipient was placed under general anaesthesia using isofluorane gas (4% at 1.5 L O₂/min for induction and maintained at 2%). The recipient was placed in a supine position and its neck was prepared for surgery and sterilely draped. A 4.0-6.0 cm incision was made over the right sternocleidomastoid muscle, and the external jugular and the common carotid artery were exposed and dissected free. 100 U/kg of heparin was given I.V. via the external jugular vein. The external jugular vein and the common carotid were clamped off using bulldog vascular clamps at the proximal ends of the vessels. The external jugular and the common carotid were transected at an angle at the distal end of the vessels. The common carotid was threaded through the lumen of a 1 cm segment of a 14-gauge Angiocath, and the external jugular was threaded through the lumen of a 1 cm segment of a 12-gauge Angiocath. Each vessel was then stretched back over its Angiocath segment, and secured with one tie of 3-0 silk at the distal end of the cuff and a 3-0 silk tie at the proximal end of the cuff. The donor rabbit was placed in a supine position under halothane gas and the heart

was exposed via a median sternotomy. Heparin at 100 U/kg was administered intravenously into the inferior vena cava (IVC). The vena cavae and the ascending aorta were cross clamped and standard cold cardioplegia solution (5-10 mL) was injected into the root of the ascending aorta proximal to the cross clamp. The thoracic cavity was then flooded with saline slush. Once asystole was achieved, the IVC and superior vena cava (SVC) were ligated and cut distally. The ascending aorta and pulmonary artery were transected and pulmonary veins were collectively tied. The heart was removed and placed in a saline slush. Retrograde perfusion of the heart with 20 ml of cold cardioplegia solution was achieved via the aorta. The graft heart was attached to the recipient rabbit's circulation by inserting the rabbit's common carotid to the graft's ascending aorta and the recipient's jugular to the graft's pulmonary artery. The anastomosis was achieved by using a 3-0 silk tie positioned in between the two ties used to secure the cuff onto the rabbit's vessels. Care was taken to ensure that neither the graft nor vessels were twisted. Once the graft was in place, the graft was de-aired using a 25-gauge needle and a 10 cc syringe. Upon re-perfusion of the graft's coronary system, the heart contractility was established and the skin was closed.

Animals were examined twice daily (every 12 h). The cervical allografts were palpated, an assessment of the strength and rate of contraction was noted. A graft was considered to have rejected if it ceased to beat, at which point it was removed and was sent for histopathological evaluation. However,

if a heart graft ceased to beat within 72 h of surgery it was considered a technical failure and was eliminated from the study. The procedures followed were in accordance with the guidelines established for the Canadian Council on Animal care in consultation with a veterinarian.

c. Drug administration in transplant recipients

MMF was obtained as a gift from Syntex (Palo Alto, CA, USA). The drug was administered using a vehicle of sodium chloride (0.9% w/v), sodium carboxymethylcellulose (0.5% w/v), polysorbate 80 (0.4% w/v), benzyl alcohol (0.9% w/v), and distilled water (97.3%). The animals were randomly divided into five treatment groups. The groups received either vehicle or drug administered as a SC dose every 12 hours (BID) as follows: group 1 vehicle control; group 2 (40 mg/kg/d); group 3 (80 mg/kg/d); and group 4 (160 mg/kg/d). Drug was administered BID from the day prior to surgery (day-1) until the day of rejection. Group number 5 received 240 mg/kg/d from day -1 up to 14 days post-transplant. The dose was subsequently dropped to 80 mg/kg/d for another 7 days after which the drug was discontinued.

d. Source of specimens for transplant studies

Trough whole blood samples were collected from recipient rabbits one day prior to surgery, the day after surgery, and every three days thereafter prior to drug administration. All samples were collected in tubes containing EDTA as an anticoagulant. The samples for IMPDH activity were stored at 4°C for a maximum of 72 hours until analysis. Enzyme activity was shown to be stable

for this period of time (Chapter VII). For determination of MPA concentrations plasma was separated and stored at -20°C until the time of analysis.

e. Drug measurement

Drug quantification in plasma was by HPLC as previously described in Chapter VI.

f. Determination of IMPDH activity

The activity of IMPDH in intact lymphocytes was measured using the procedure previously described in Chapter VII.

g. Pharmacokinetic and statistical analysis

Plasma concentration versus time data for MPA following administration of MMF were fitted to a non-compartmental model using PCNONLIN V 4.0 (Statistical Consultants Inc., Lexington, KY, USA). A total of 13 parameters are calculated by this program, including β half-life ($t_{1/2\beta}$), area under the curve (AUC), and mean residence time (MRT). From the fitted parameters, other off-line pharmacokinetic parameters were calculated using the following equations:

$$\text{Apparent clearance} \\ (Cl) = \frac{\text{Dose}}{\text{AUC}}$$

$$\text{Apparent volume of distribution at steady state} \\ (V_{D_{ss}}) = \frac{(\text{Dose})(\text{AUMC})}{(\text{AUC})^2}$$

Differences between pharmacokinetic parameters were assessed with unpaired t-tests using SPSS statistical software (SPSS Inc. Chicago, IL). A p value <0.05 was considered significant.

3. RESULTS

a. Pharmacokinetic parameters in the dog

The pharmacokinetic parameters obtained are summarized in Table VIII-1. Although, the C_{max} of the 40 mg/kg group (31.6 ± 15.2 mg/L) was statistically greater than the 20 mg/kg group (12.8 ± 7.4 mg/L) ($p < 0.05$), there were no statistical differences in the other parameters investigated (AUC, $t_{1/2}$, MRT, VD_{ss} , and Cl) between the two treatment groups. A second peak of MPA in plasma is observed in the dogs at between 8 and 12 h post-dose. This phenomenon is most likely due to enterohepatic circulation of MPA.

b. Pharmacokinetic parameters in the rabbit

The pharmacokinetic parameters obtained from the rabbits are summarized in Table VIII-2. In rabbits administered MMF at a dose of 40 mg/kg IV the $t_{1/2}$ in the rabbit was 0.61 h. This is similar to what has been reported in other studies (217). The AUC, Cl, MRT, and VD_{ss} were also similar to that previously reported (217). When the rabbits were administered a dose of 80 mg/kg SC of MMF the half life (0.78) was similar to the IV route as described above. In the 80 mg/kg SC group the VD_{ss} , MRT, and Cl were not statistically different from the 40 mg/kg IV group; the AUC was higher in the former, but this was expected since the dose was greater. After 7 days of SC administration the pharmacokinetic parameters were re-evaluated and there was no statistical difference from day 0 to day 7.

Table VIII-1

Pharmacokinetic parameters in the canine following a single oral dose of MMF

A. 20mg/kg PO

Dog	$t_{1/2}$ (h)	AUC (mg.h/L)	Cl (L/h/kg)	VDss (L/kg)	MRT (h)
1	6.33	15.4	0.141	11.9	9.18
2	12.92	154.7	0.007	2.4	18.38
3	12.74	43.3	0.021	9.8	21.12
4	7.32	64.1	0.027	3.6	11.41
5	1.45	66.8	0.033	2.7	8.99
6	8.05	29.6	0.081	5.6	8.26
mean	8.1	62.3	0.052	5.9	12.9
SD	3.9	45.0	0.046	3.6	5.0

B. 40mg/kg PO

Dog	$t_{1/2}$ (h)	AUC (mg.h/L)	Cl (L/h/kg)	VDss (L/kg)	MRT (h)
1	11.09	104.2	0.037	3.9	10.22
2	1.25	126.4	0.065	1.5	4.85
3	3.75	77.3	0.091	3.0	5.69
4	9.30	42.7	0.067	13.0	13.93
5	9.42	96.6	0.401	0.4	10.35
6	2.15	48.7	0.148	4.6	5.55
mean	6.2	82.6	0.135	4.4	8.43
SD	3.9	29.8	0.123	4.1	3.31

Table VIII-2

Pharmacokinetic parameters in rabbits following a single dose of MMF.

A. 40 mg/kg IV

Rabbit	Tmax (h)	AUC (mg.h/L)	Cl (L/h/kg)	VDss (L/kg)	t _{1/2} (h)
1	0.25	18.32	2.18	1.65	0.52
2	0.25	29.62	1.35	1.36	0.65
3	0.25	20.75	1.93	1.61	0.61
4	0.25	18.01	2.22	1.94	0.64
mean	0.25	21.68	1.92	1.64	0.61
SD	0.00	4.71	0.35	0.20	0.05

B. 80 mg/kg SC

Rabbit	Tmax (h)	AUC (mg.h/L)	Cl (L/h/kg)	VDss (L/kg)	t _{1/2} (h)
1	0.50	52.2	1.53	1.52	0.54
2	0.50	50.0	0.77	0.83	0.58
3	1.00	56.5	0.38	1.29	1.15
4	0.50	55.7	0.50	1.04	0.85
mean	0.63	53.6	0.80	1.17	0.78
SD	0.22	2.6	0.45	0.26	0.25

c. Pharmacodynamic analysis in the dog

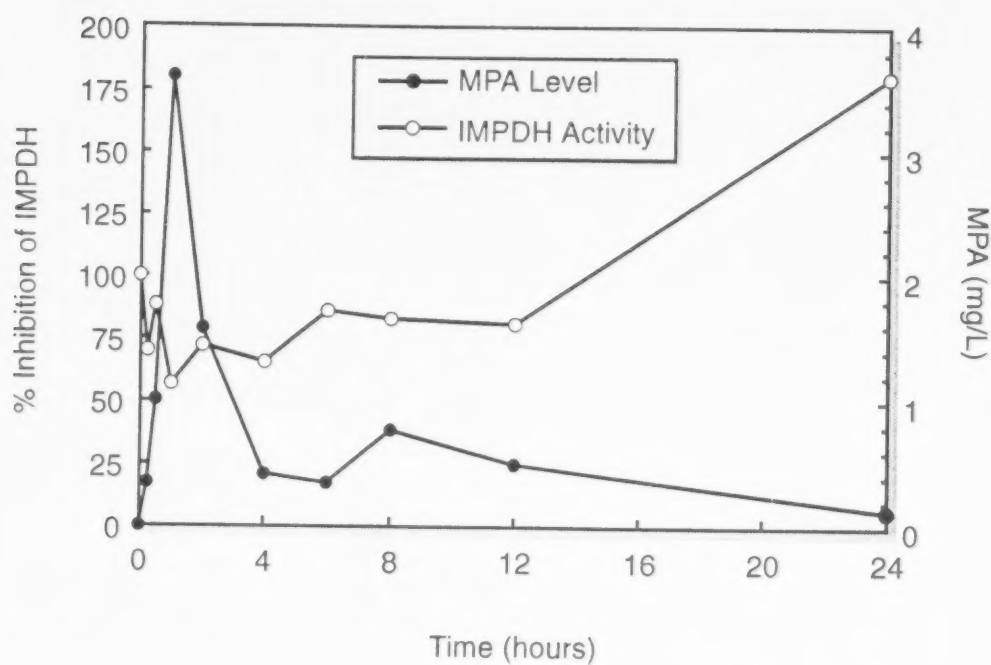
When individual dogs were analyzed an inverse relationship was observed between drug concentrations and IMPDH activity. A typical example of individual animals responses are shown in Figure VIII-1A and Figure VIII-1B for the 20 mg/kg and 40 mg/kg doses, respectively. When data from all dogs were combined, a large variation (as indicated by the large SE) for both MPA concentration and IMPDH activity was observed as shown for the two doses investigated (Figure VIII-2A and VIII-2B). The peak MPA level was attained at approximately 2 hours post dose; the maximum inhibition of IMPDH activity, of 20 to 25%, occurred slightly later at 4 h post-dose. There does not appear to be a dose dependent inhibition of IMPDH activity. As the MPA concentration decreases throughout the dosing interval, there is a gradual restoration of enzyme activity and at 24 hours enzyme activity exceeds the pre-dose enzyme activity. In some of the dogs, the 24 hour post dose IMPDH activity exceeds the pre-dose levels by as much as three fold, with no change in the WBC. There was no significant correlation with AUC and the maximum % inhibition of IMPDH ($r=0.01$), AUC and the duration of inhibition ($r=0.42$), C_{max} and the maximum % inhibition of IMPDH activity ($r=0.09$), and C_{max} and the duration of inhibition ($r=0.06$). At 24 hours post dose the mean IMPDH activity exceeds that of the pre-dose values.

d. Pharmacodynamic analysis in the rabbit

The results shown in Figure VIII-3 indicate an inverse relationship

Figure VIII-1A.

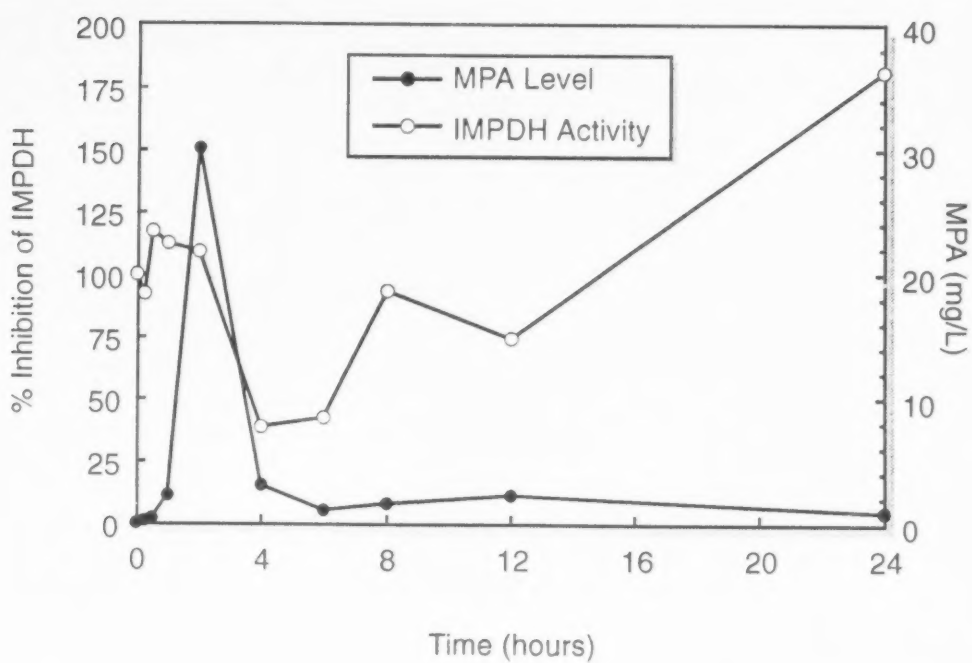
Pharmacokinetic/Pharmacodynamic profile of a dog receiving 20 mg/kg of MMF



PK/PD profile of a representative dog receiving a single oral dose of 20 mg/kg of MMF. MPA concentration and IMPDH activity throughout a 24 h dosing interval. The IMPDH activity of the pre-dose sample was 185.3 dpm/min.

Figure VIII-1B

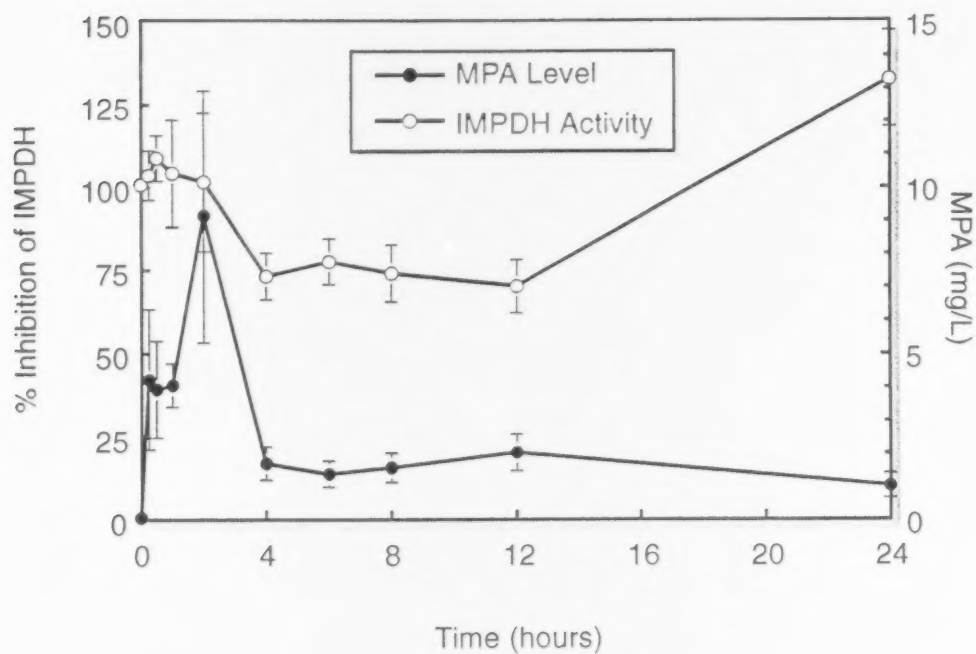
Pharmacokinetic/Pharmacodynamic profile of a dog receiving 40 mg/kg of MMF



PK/PD profile of a representative dog receiving a single oral dose of 40 mg/kg of MMF. MPA concentration and IMPDH activity throughout a 24 h dosing interval. The IMPDH activity of the pre-dose sample was 244.4 dpm/min

Figure VIII-2A.

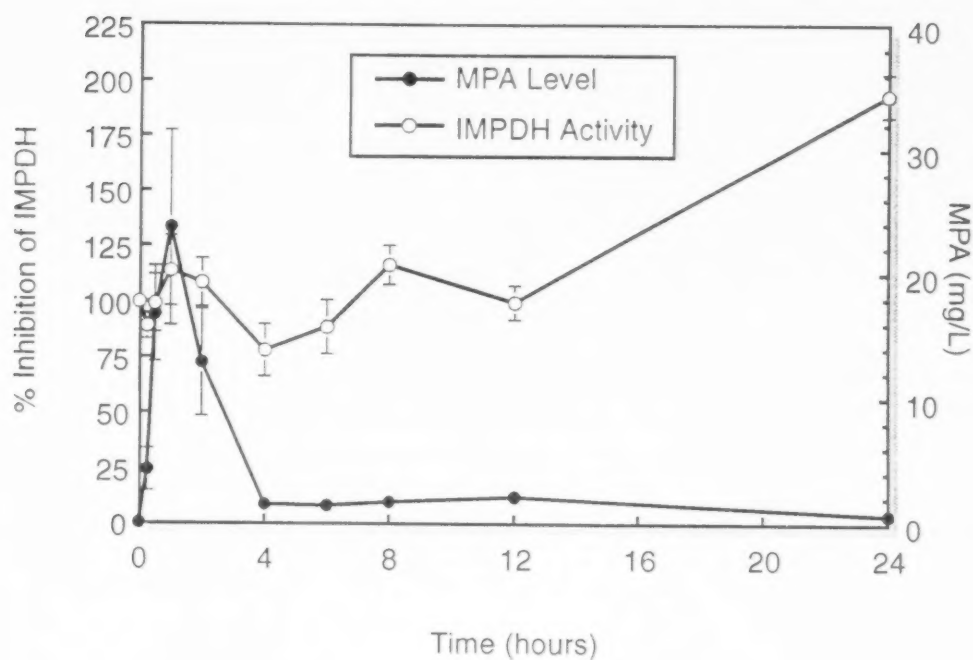
Mean Pharmacokinetic/Pharmacodynamic profiles of dogs receiving 20 mg/kg of MMF.



PK/PD profiles of dogs receiving a single oral dose of 20 mg/kg of MMF. Mean MPA concentrations and IMPDH activity throughout a 24 h dosing interval are shown. The results are expressed as mean $\pm$ SE ($n=6$). The mean IMPDH activity of the pre-dose samples was 223.0 ± 90.1 dpm/min.

Figure VIII-2B.

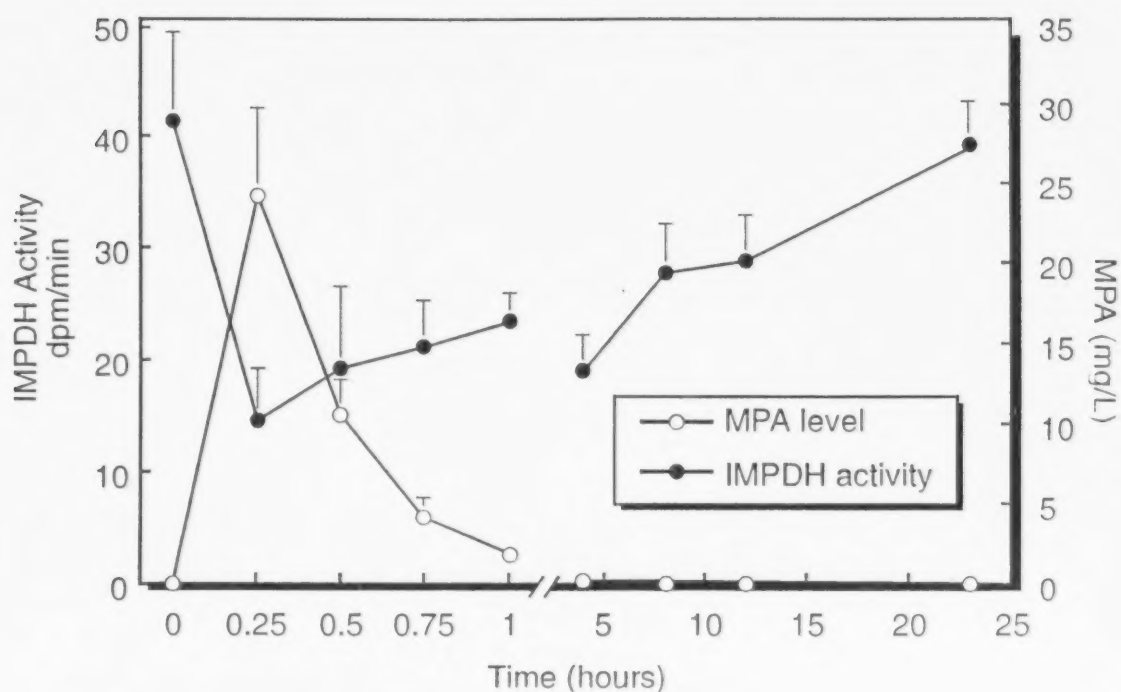
Mean Pharmacokinetic/Pharmacodynamic profiles in dogs receiving 40 mg/kg of MMF.



PK/PD profiles of dogs receiving a single oral dose of 40 mg/kg of MMF. Mean MPA concentrations and IMPDH activity throughout a 24 h dosing interval are shown. The results are expressed as mean $\pm$ SE (n=6). The mean IMPDH activity of the pre-dose samples was 152.9 ± 55.7 dpm/min.

Figure VIII-3

Mean Pharmacokinetic/Pharmacodynamic profiles in rabbits receiving 40 mg/kg of MMF.



PK/PD profiles of rabbits receiving a single IV dose of 40 mg/kg of MMF. Mean MPA concentration and IMPDH activity throughout a 24 h dosing interval are shown. The results are expressed as mean $\pm$ SE (n=4).

between drug concentrations and IMPDH activity. The peak concentrations (C_{max}) of MPA achieved at 0.25 h resulted in approximately 60% inhibition of IMPDH activity which is similar to that observed for the *in vitro* studies where approximately 70% inhibition was observed (Chapter VII). As the MPA concentration decreases throughout the dosing interval, there is a gradual restoration of enzyme activity and at 24 hours enzyme activity is completely restored to pre-dose levels.

When the drug was administered SC a similar phenomenon was seen. On day 0 the peak drug level was attained at 30 min post-dose, which corresponds to the maximal inhibition of IMPDH activity (Figure VIII-4A). After 7 days of drug administration, C_{max} was achieved at 15 min post dose, and the maximal inhibition of IMPDH activity was attained at this time (Figure VIII-4B). However, there was no statistical difference as compared to day 0.

e. Graft survival in heterotopic heart transplants

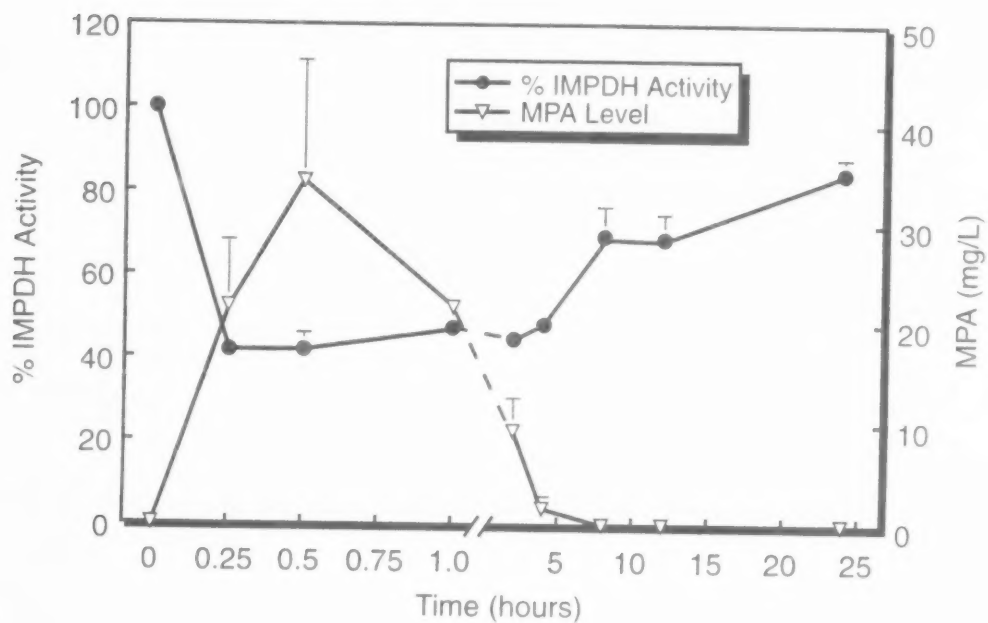
The graft survival of each of the dose groups are indicated in Figure VIII-5 and Table VIII-3. The control animals had an average survival of approximately 10 days. The 80 mg/kg/d dose group had an average graft survival of 14.8 ± 4.2 days, which was not statistically significant from controls ($p = 0.058$). However, statistically significant prolongation of graft survival was achieved with a dose of 160 mg/kg/d ($p = 0.005$).

f. Histological analysis

All rabbit donor hearts showed histological evidence of acute graft

Figure VIII-4A

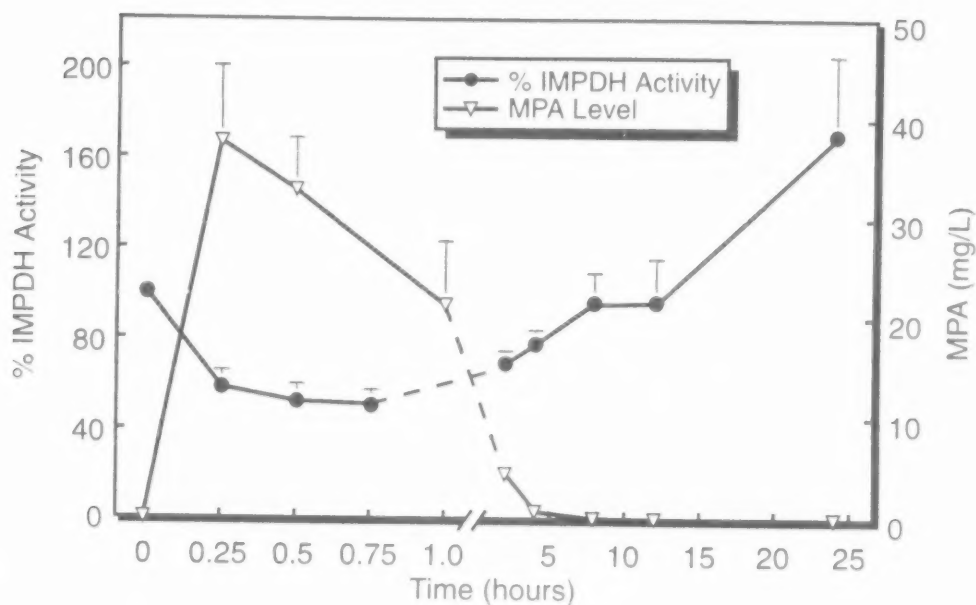
Mean Pharmacokinetic/Pharmacodynamic profiles of rabbits receiving 80 mg/kg of MMF.



PK/PD profiles of rabbits receiving a single 80 mg/kg SC dose of MMF. Mean MPA concentration and IMPDH activity throughout a 24 h dosing interval on day 0 are shown. The results are expressed as mean $\pm$ SE ($n=4$). The mean IMPDH activity in pre-dose samples was 28.7 ± 1.0 dpm/min.

Figure VIII-4B

Mean Pharmacokinetic/Pharmacodynamic profiles in rabbits receiving 80mg/kg of MMF.



PK/PD profiles of rabbits receiving a single 80 mg/kg SC dose of MMF. Mean MPA concentration and IMPDH activity throughout a 24 h dosing interval after 7 days of MMF administration are shown. The results are expressed as mean $\pm$ SD ($n=4$). The mean IMPDH activity in samples pre-dose was 26.0 ± 4.1 dpm/min.

Figure VIII-5

Kaplan-Meyer plot of graft survival in rabbit heterotopic heart transplant recipients receiving MMF.

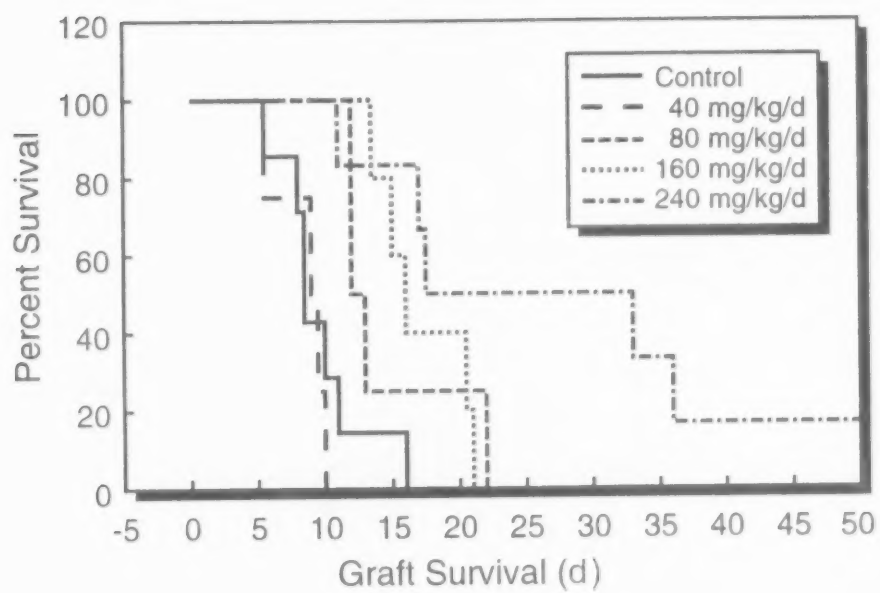


Table VIII-3

Graft survival in rabbit heterotopic heart transplant recipients receiving mycophenolate mofetil (MMF).

Dose of MMF	n	p versus control	Graft Survival (d)
control	7	-	9.6 ± 3.0
40 mg/kg/d	4	0.549	8.5 ± 1.8
80 mg/kg/d	4	0.058	14.8 ± 4.2
160 mg/kg/d	5	0.005	17.1 ± 3.1
240 mg/kg/d ¹	6	0.010	27.4 ± 13.5

1 - dose was decreased to 80 mg/kg/d after day 14 and discontinued after day 21.

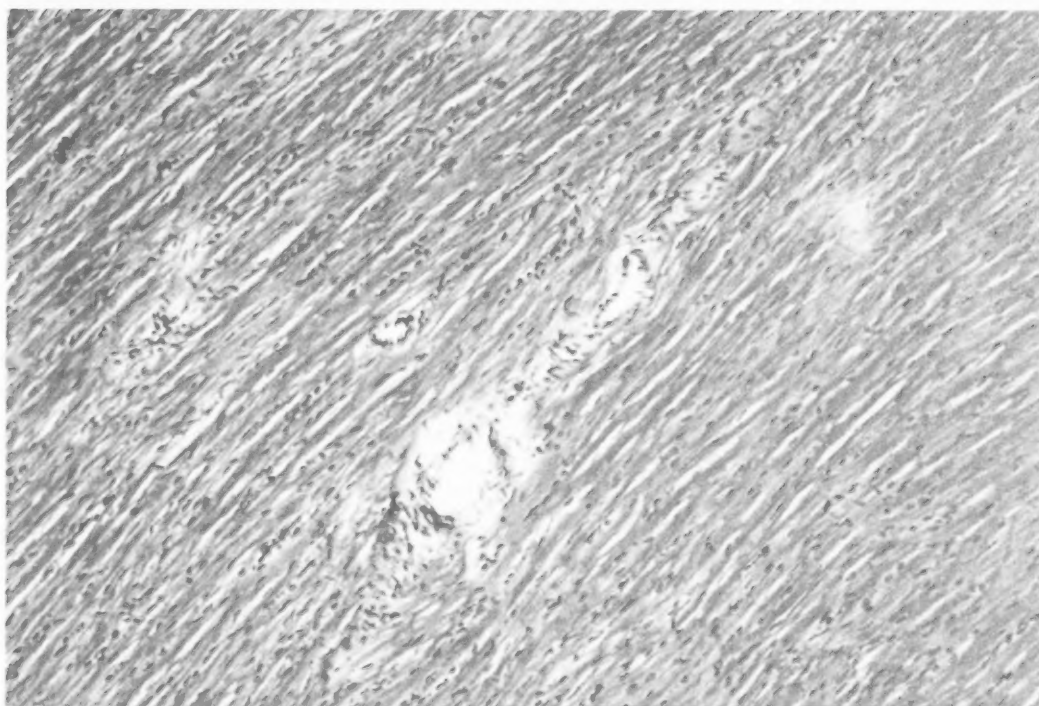
rejection, as outlined by the International Society of Heart Transplantation (232), at the time of cessation of beating. The results are expressed in Table VIII-4. All of the animals showed high rejection scores at the time the hearts stopped beating. The heart of one rabbit from the 240 mg/kg/d dose group continued to beat after 50 days post-transplant. The ISHT rejection score in this heart was 3b. The median grade of rejection in all the donor hearts was 3b. The histology of normal rabbit cardiac tissue is shown in Figure VIII-6, and characteristic features of grade 3b rejection (diffuse inflammatory processes, myocyte damage, aggressive inflammatory infiltration of large lymphocytes, and eosinophils with an occasional neutrophil) are shown in Figures VIII-7 to VIII-9. Figure VIII-7 shows an extensive lymphocytic infiltration, myocyte damage, and areas of fibrotic tissue (232). Figure VIII-8 shows lymphocytic invasion of the vasculature and Figure VIII-9 shows eosinic infiltration.

g. Correlation analysis

The highest dose group (Group 5, 240 mg/kg/d) underwent dosage adjustments at two time points in the course of their treatment. Therefore, it may be inappropriate to include this group in the remainder of the statistical analysis. The % IMPDH activity versus the time post transplant was plotted for individual animals, as shown in Figures VIII-10 and VIII-11. In the control animals (Figures VIII-10) there was little change in the IMPDH activity immediately post treatment. In most animals treated with MMF there was a decrease in IMPDH activity in the days following the transplant (Figure VIII-11).

Figure VIII-6

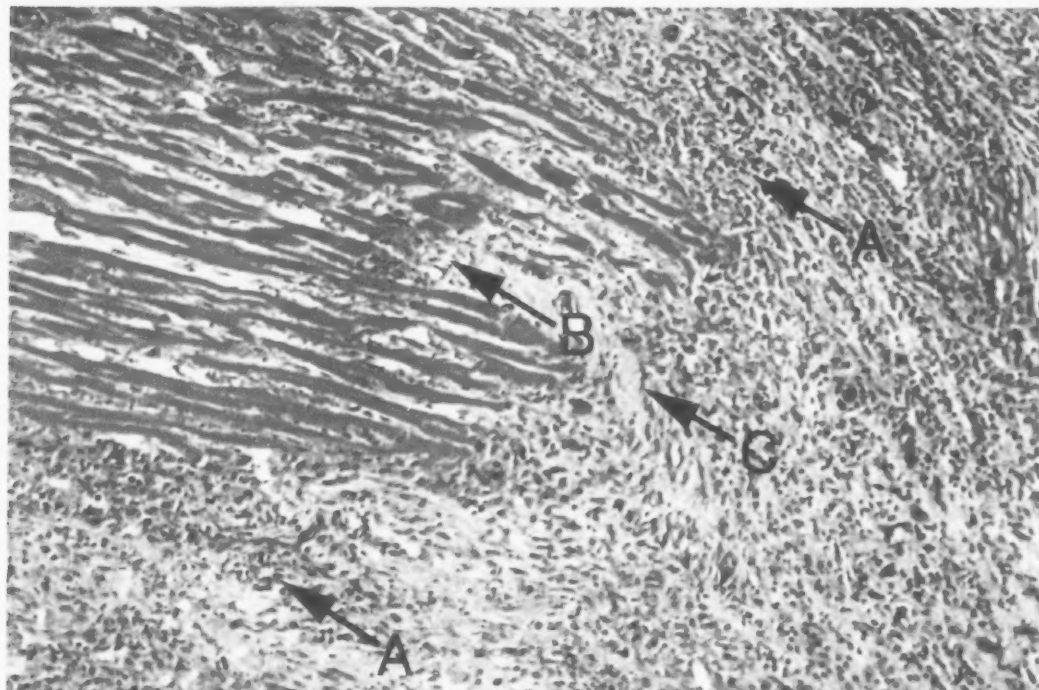
Photomicrograph of normal rabbit myocardium



Photomicrograph of normal rabbit myocardium. Hematoxylin and Eosin (H&E) stain.
(original magnification X40)

Figure VIII-7

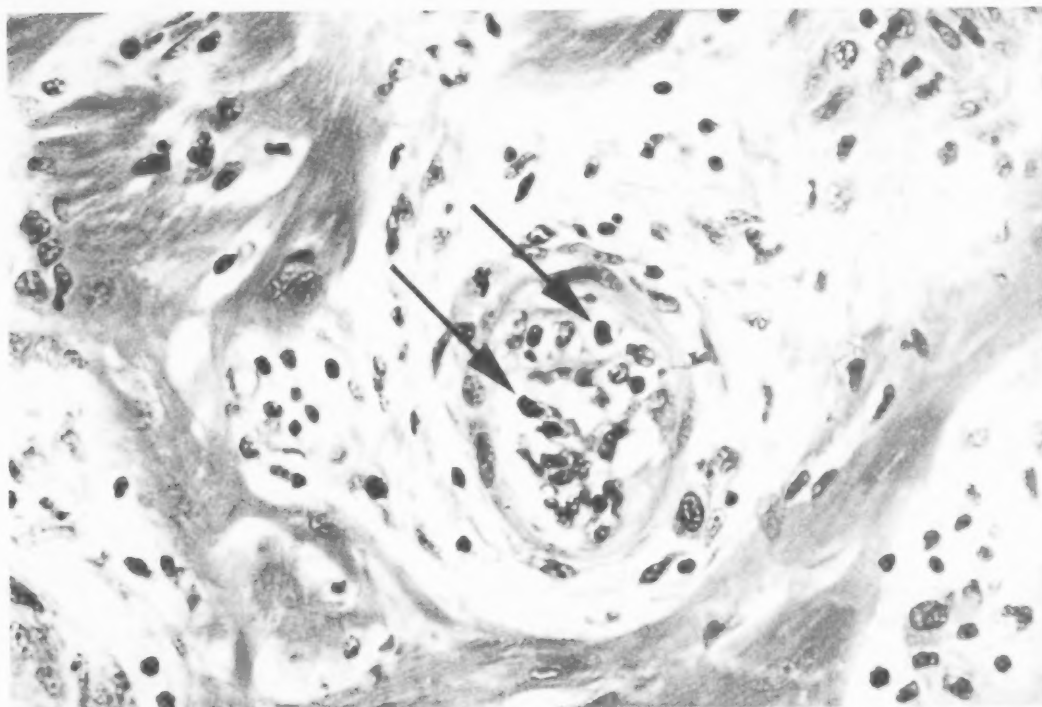
Photomicrograph of International Society of Heart Transplantation (ISHT) grade 3b rejection.



Photomicrograph of ISHT grade 3b rejection. H&E stain indicating lymphocytic infiltrate (A), myocyte damage (B), and interstitial fibrosis (C). (original magnification X40)

Figure VIII-8

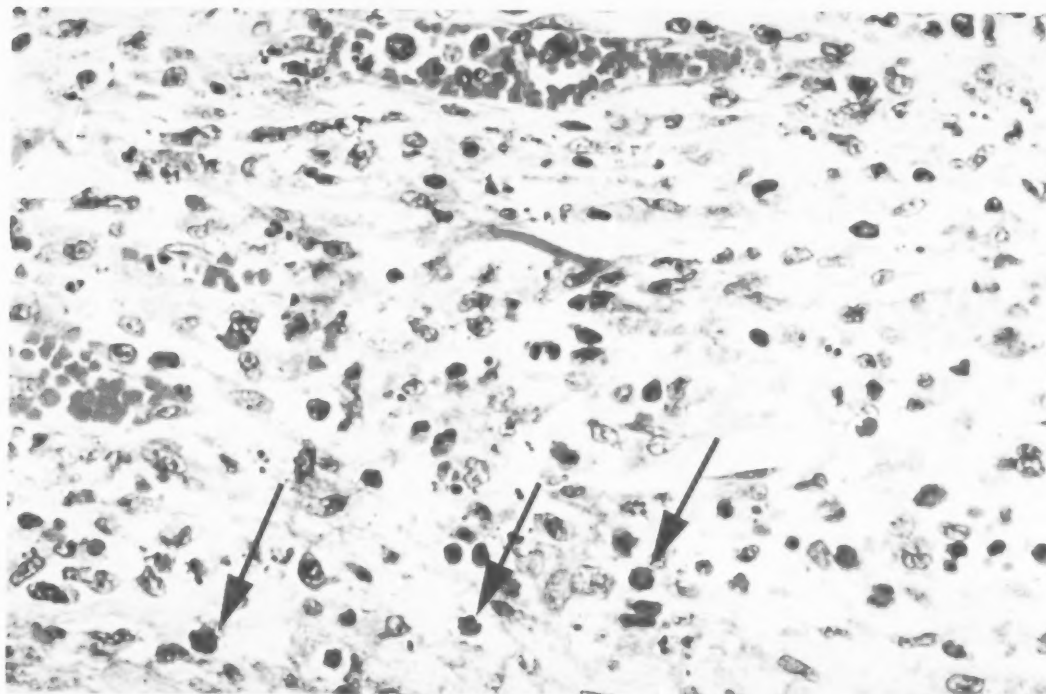
Photomicrograph of International Society of Heart Transplantation (ISHT) grade 3b rejection.



Photomicrograph of ISHT grade 3b rejection. H&E stain indicating lymphocytic infiltration of the vasculature as indicated by the arrows. (original magnification X150)

Figure VIII-9

Photomicrograph of International Society of Heart Transplantation (ISHT) grade 3b rejection.



Photomicrograph of ISHT grade 3b rejection. H&E stain indicating eosinophilic infiltrate as indicated by the arrows. (original magnification X150)

Table VIII-4

International Society of Heart Transplantation (ISHT) rejection score by dose group

Group	Rejection Score ¹
control	3a, 3b, 3b, 3b, 3a, 3b, 3b,
40 mg/kg/d	3b, 3b, 3b, 4
80 mg/kg/d	4, 4, 1b, 3b
160 mg/kg/d	3b, 4, 4, 3b, 3 ²
240 mg/kg/d ²	3b, 3a, 3b, 3b, 3a, 3b

1- See table below for description

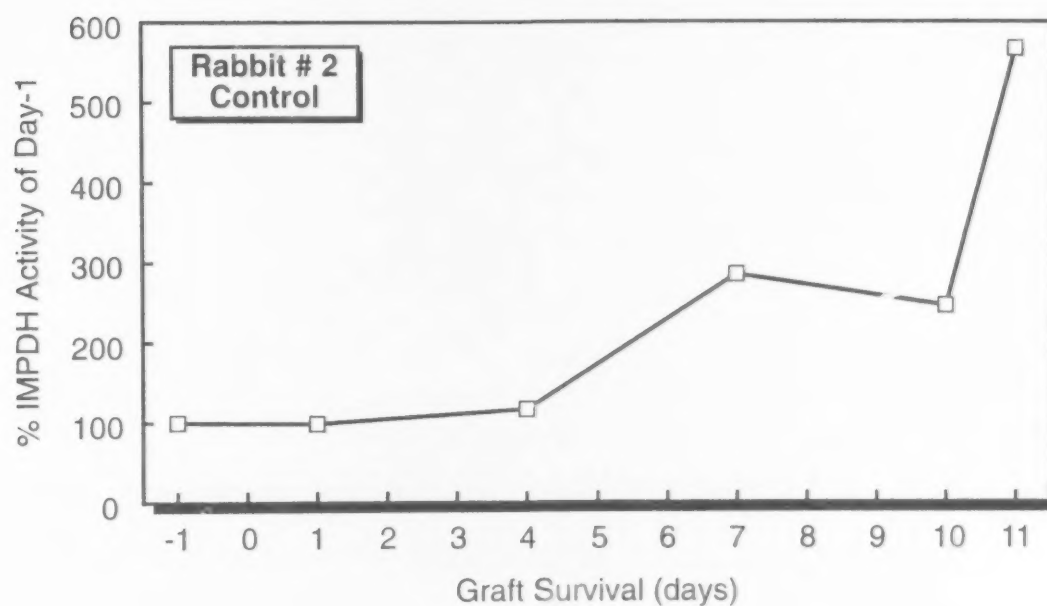
2- dosage was decreased to 80 mg/kg/d after 14 days and discontinued after day 21.

ISHT grading criteria

Grade	Features
0	no rejection
1	a. focal mild acute rejection. b. diffuse mild rejection
2	focal moderate rejection
3	a. multi-focal moderate rejection b. diffuse borderline severe acute rejection
4	severe rejection

Figure VIII-10

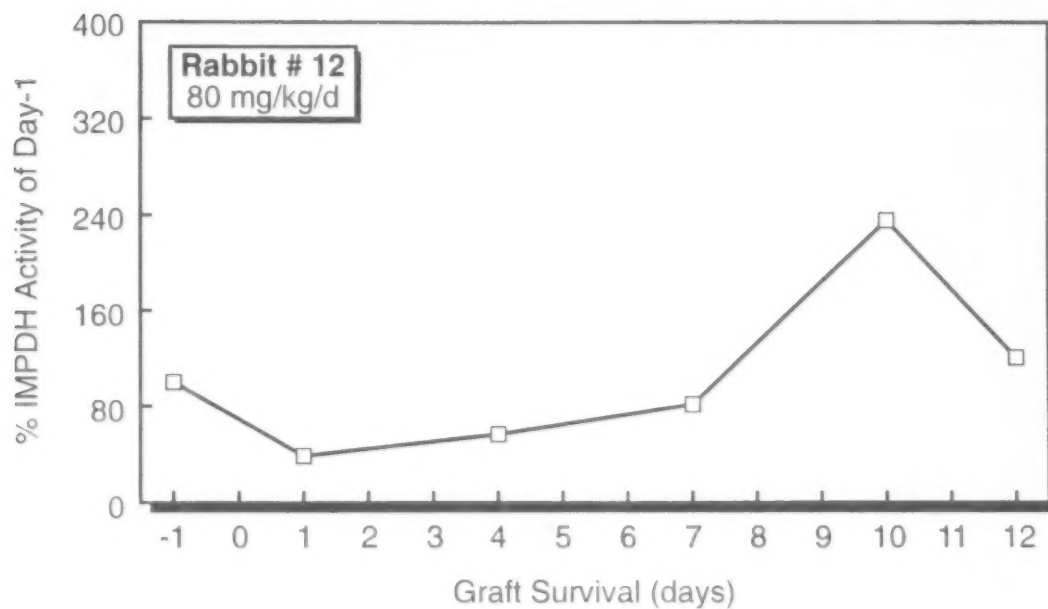
IMPDH activity versus days post-transplant in control (not receiving MMF) animal #2.



Change in IMPDH activity, expressed as % of day-1, as it relates to cardiac allograft survival in a rabbit. WBC count did not change significantly over the treatment interval.

Figure VIII-11

IMPDH activity versus days post-transplant in a MMF treated animal receiving 80 mg/kg/d SC, animal #12.



Change in IMPDH activity, expressed as % of day-1, as it relates to cardiac allograft survival in a rabbit. WBC count did not change significantly over the treatment interval.

In approximately 80% of the cases, in both control and treated groups, there was an increase in IMPDH activity a few days prior to rejection, with an average maximum increase of 96.5% above the enzyme activity pre-transplant.

The mean MPA concentration, IMPDH activity and graft survival for each treatment group, were compared using a linear regression model (Table VIII-5). Significant correlations ($p \leq 0.05$) were seen with MPA concentration versus dose and graft survival, with correlation coefficients (r) of 0.98 and 0.94, respectively. A negative correlation between IMPDH activity and the MPA concentration ($r = -0.83$), and between the former and the average graft survival in each dose group ($r = -0.63$) was obtained (Table VIII-5). The multivariate relationship between MPA concentration in plasma, % IMPDH activity, and graft survival is shown in Figure VIII-13. The data also indicates that as the drug concentration increases the IMPDH activity decreases with a corresponding increase in graft survival.

4. DISCUSSION

The canine model has been widely used for the investigation of immunosuppressive drugs, but dogs may be more prone to systemic side effects than other species. Intestinal toxicity is often the limiting side-effect manifesting itself as severe diarrhoea and malnutrition (82,231). These side effects can be minimized by a decrease in the dosage of the drugs, however, this could compromise graft survival. In the past the most common MMF

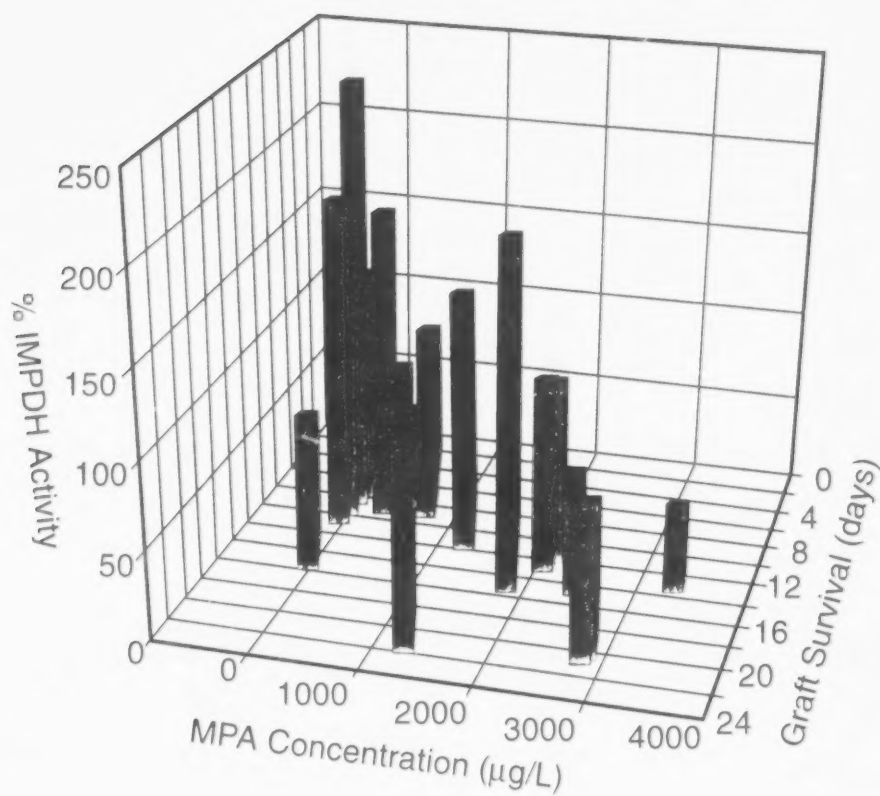
Table VIII-5.

Multivariate analysis of graft survival, dose, MPA concentration, and IMPDH activity

X	vs	Y	r =	p =
MPA concentration		dose	0.98	0.02
% IMPDH activity		dose	-0.86	0.14
graft survival		dose	0.90	0.10
MPA concentration		% IMPDH activity	-0.83	0.17
graft survival		MPA concentration	0.95	0.05
graft survival		% IMPDH activity	-0.63	0.37

Figure VIII-13

Multivariate relationship between MPA concentration, %IMPDH activity, and graft survival for individual animals.



dosages in dogs have been 20 and 40 mg/kg/d in a single dose. To assess the appropriateness of these doses and dosing intervals we related the pharmacokinetic properties of the drug to its biological effect (inhibition of IMPDH activity) to predict the most beneficial dosing regime.

An evaluation of the pharmacokinetic data showed there is considerable variation among dogs in absorption and clearance. In the canine model the $t_{1/2}$ of MPA is approximately 8 h, which is considerably longer than the rabbit model (0.61 h) but comparable to that previously reported in humans (233). The canine model also revealed a second peak in the MPA plasma levels following single dose administration. This is probably due to enterohepatic circulation. The second MPA peak is not detectable in the rabbit model, but the rapid clearance of MPA and large gaps between blood sampling times could mask the appearance of this phenomenon. In dogs there was substantial variation in the absorption and clearance of MPA among the animals tested. This was not unexpected, as substantial variation among humans has also been noted (233). From the data presented here an appropriate dosing interval for maintenance therapy in the dog can be approximated. The dosing interval should approximate the half-life of the drug, if the goal of therapy is to minimize plasma fluctuations to no more than 50% between doses (234). Therefore, an 8 h interval would be recommended.

The relationship of MPA concentration to IMPDH inhibition *in vivo* throughout a 24 hour dosing interval was evaluated in dogs and rabbits. The

results indicate an inverse relationship between IMPDH activity and MPA concentration. As the concentration of the drug decreases post-dose, there is a gradual increase in IMPDH activity. In the dog the maximal inhibition of IMPDH activity occurs approximately 2 to 4 hours subsequent to the peak plasma concentration. In the rabbit, however, the peak concentration of drug and the maximum inhibition of IMPDH activity occur at the same time. The discordance between peak drug concentration and maximal IMPDH inhibition in the canine model could be due to differences in the uptake of MPA into target lymphocytes. Previous studies *in vitro* (Chapter (VII)) showed that the effect of MPA on IMPDH activity was immediate, however, this study was performed in rabbit and human whole blood. If canine lymphocytes have a slower mechanism for MPA uptake than rabbit lymphocytes, more time would be needed for intracellular MPA concentrations to reach a level sufficient to inhibit IMPDH. This may also explain why higher concentrations are needed for inhibition in the canine model.

The inhibition of IMPDH activity by MPA *in vitro*, in whole blood, is consistent with that *in vivo* for both the canine and rabbit models. In the dog the peak MPA concentrations of 120 mg/L results in an inhibition of approximately 30%. In the rabbit maximal inhibition of IMPDH activity of approximately 60% occurs at peak concentrations of MPA; as the concentration of the drug decreases post dose, there is a gradual increase in enzyme activity with little or no inhibition being observed at 24 hours. In

contrast, in the canine model after 24 hours post-dose the IMPDH activity increased, in some cases up to three times greater than pre-dose levels. This provides further support for the use of a BID or TID dosing regimes in an attempt to prevent the apparent increase of enzyme activity seen after single dose administration of MMF in the dog. The reason for the latter is not known, and further investigation is required. However, in other studies, MPA has been shown to cause an increase in IMPDH gene transcription, due to depletion of guanine nucleotides (119,120). If dogs respond more quickly or to a greater extent than do other species to altered guanine nucleotide pools, the increase in IMPDH activity could be due to newly synthesized enzyme. The effect of chronic drug administration on IMPDH activity or gene transcription in this model has not yet been investigated.

The effect of chronic SC administration in the rabbit was investigated. The pharmacodynamic response to MPA appears to be similar to that observed with IV administration. Peak MPA levels appear at time point, 30 min as compared to 15 min, since it requires time for the drug to be absorbed and enter the blood stream. However, the inverse relationship between MPA concentration and IMPDH activity, as described above, is similar. The degree of inhibition and the duration of inhibition post-dose are comparable. The results after 7 days of treatment SC are not statistically different from those obtained on day 0. This suggests that the response after chronic administration is the same as after acute administration, allowing a certain degree of

predictability in the level of response to a given dose.

MPA has been shown to be effective in the prolongation of allograft survival in the animal models (237,82,231). Although inhibition of IMPDH is transient and not complete, it may be sufficient to alter the balance of purine nucleotides within lymphocytes, resulting in impairment of DNA synthesis and in the synthesis of glycoproteins involved in the adhesion process. This is analogous to that observed for CsA, which at therapeutic concentrations *in vivo* results in only a 50% inhibition in CN activity (235,236). It is unknown what level of inhibition of IMPDH is required for sufficient alteration of cellular nucleotide levels to achieve the desired immunosuppressive effect. The different responsiveness to MPA in dogs versus the human and rabbit *in vitro* could reside within the IMPDH molecule itself. The amino acid sequences of IMPDH in dogs could be sufficiently different, from rabbit and human, that the susceptibility to MPA is different, but no less effective in preventing rejection. There is also little published data regarding the different isoforms of IMPDH in the rabbit or dogs. The sensitivity of these isoforms to MPA is not characterized. In humans, IMPDH exists in two isoforms: type I and type II (41). During lymphocyte stimulation there is an up regulation of the type II isoform with no change in the type I isoform (41). The type II isoform has been shown to be 3.9 fold more sensitive to MPA than the type I isoform (41). It would be reasonable to assume there would be a comparable situation in dogs. The inducible form of IMPDH could be more sensitive to MPA and therefore in

an immune stimulated animal lower levels of MPA are more effective, thus preventing rejection at low doses of drug. However, if the increase in type II isoform is sufficiently large much greater levels of MPA would be required to result in adequate enzyme inhibition. Alternatively, the change in glycoproteins involved in cellular adhesion, which was not measured here, may also contribute to overall immunosuppression.

The combination of PK and PD studies to evaluate the appropriateness of dosing regimes is an approach that can be used for other animal models. In previous *in vitro* and *in vivo* PD studies it was found that the rabbit most closely resembles the human in MPA induced inhibition of IMPDH activity. However, the rabbit model has a very rapid clearance of MPA, with a half life ($t_{1/2}$) of less than an 1 h, while humans have a $t_{1/2}$ of approximately 8 hours. PD studies have suggested that dosing of MMF could be optimized at every 12 h for both species, and therefore was used as the dosing interval.

The relationship between versus drug level monitoring was evaluated in a rabbit heterotopic heart transplant model since the transplant procedure is relatively simple to perform and, due to the placement of the heart in the neck of the animal, allows for easy monitoring of graft function. Studies in other animal models have shown doses of 40 mg/kg/d to be effective in the prevention of allograft rejection when used in combination with other immunosuppressive agents (237,82,231). We chose to evaluate the effectiveness of MMF over a higher range of doses from 40 mg/kg/d to 240

mg/kg/d due to its rapid clearance and its use as the sole immunosuppressive agent. A dose dependant increase in graft survival was observed. Although graft survival is significantly greater with MMF treatment at 160 mg/kg/d, long term graft survival was not seen. MPA inhibits only one step in the sequence of events that occur after the activation of the T-cell through the T-cell Receptor (TCR)/CD3 complex. It is apparent that MMF alone is not sufficient to prevent the activation of T-cells or their effector function and, therefore, would more likely be effective when used in conjunction with other immunosuppressive agents. It has been shown that the combinations of MMF with other immunosuppressive drugs, such as CsA, are more effective (27,82,231).

MMF was recently approved by the US Food and Drug Administration for the prevention of rejection in renal transplant patients (239). The role of TDM for a therapeutic strategy has not been thoroughly evaluated. It remains to be determined if either total MPA concentration or free fraction, would best relate to drug induced immunosuppression. There have been no published reports on the therapeutic range for MPA in plasma. A proportionality exists between dose and concentration for many drugs. This appears to be the case with MMF in the rabbit. Significant prolongation of graft survival was observed with a dose of 160 mg/kg/d, therefore, the corresponding steady state MPA trough concentration of 2.5 mg/L obtained with this dose, may approximate plasma therapeutic values.

The measurement of IMPDH activity in whole blood as previously described provides a simple reproducible assay for the biological effect of MMF. It was found that an inverse relationship between MPA concentration in plasma and IMPDH activity exists *in vitro* and *in vivo*. The extent of the MPA mediated inhibition of IMPDH activity *in vivo* are comparable to the *in vitro* data. A significant inverse relationship was noted between IMPDH activity and MPA concentration. *In vitro* studies have suggested that the free MPA concentration may more accurately reflect the biological effect in the drug than does the measurement of the total MPA (179). Thus, a stronger relationship between IMPDH activity and the free rather than the total drug concentration may exist (179). This is under investigation.

A trend to an inverse relationship between IMPDH activity and graft survival was observed. From previous pharmacodynamic studies in rabbits and humans it was shown that maximal inhibition of IMPDH activity is achieved at the time of C_{max} of MPA. Enzyme activity gradually returned to pre-dose levels after 12-24 hours post-dose. Due to limitations in the amount of blood that we were allowed to collect, we chose to monitor enzyme activity at the more commonly used time points of trough rather than peak levels. A better correlation between graft survival and IMPDH activity may have been achieved with measurement of maximal inhibition of IMPDH activity which occurs at C_{max}.

Prior to rejection, approximately 80% of the animals demonstrated an

average increase in IMPDH activity of 96.5% above the pre-transplant activity. During lymphocyte stimulation there may be an up regulation of the type II isoform with no change in the type I isoform (41). The increase in IMPDH activity noted in this study could be a reflection of the induction of the type II isoform. Since an increase in IMPDH activity appears a few days prior to rejection, measurement of IMPDH activity may have potential for use as a marker of graft rejection.

This study is the first to show a concentration effect relationship with graft survival, suggesting traditional TDM is more effective than PD monitoring in the prediction of graft survival. However, the limitations of the present study do not allow extrapolation to other transplant models. The question of whether the measurement of the biological response may provide a useful adjunct to traditional TDM for the optimization of dosing of immunosuppressive drugs, still requires further investigation.

IX. PHARMACOKINETIC AND PHARMACODYNAMIC ASSESSMENT IN RENAL TRANSPLANT RECIPIENTS

1. RATIONALE:

The PD monitoring of MMF by measurement of inhibition of IMPDH activity in whole blood has been discussed for the rabbit and canine models. Results suggest that use of PK and PD monitoring can be used to optimize MMF dosing regimes, monitor therapy, and possibly predict rejection. However, this has not been investigated in humans. MMF has recently been approved for clinical use in renal transplants. Therefore, the relationship of MPA concentrations and IMPDH activity was evaluated in this patient group.

2. MATERIALS AND METHODS

a. Source of specimens

Whole blood samples were collected from a number of normal subjects ($n = 22$) and from renal-transplant recipients enrolled in the MMF clinical trial comparing the safety and efficacy of MMF ($n = 5$), with that of AZA ($n = 7$) in the treatment of acute rejection, for a total of 12 patients. The protocol was approved by the Ethics Committee of the University of Alberta. Each subject received an oral preparation of either MMF (1 or 1.5 g bid) or AZA (1 mg/kg) in combination with CsA and prednisone. All drugs were administered at the same time using a BID dosing regime. All patients were at least 2 y

post-transplant and therefore represented a chronically-treated group. All patients had stable renal function and were clinically free of rejection at the time of the study. Blood samples were collected at the following times: Pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h post-dose. Specimens were also collected from 5 normal subjects at these times. All samples were collected in tubes containing EDTA as an anticoagulant. The samples for IMPDH activity were stored at 4°C until the time of analysis. For determination of MPA concentrations, plasma was separated and stored at -20°C until analysis.

b. Drug measurement

Drug quantification in plasma was by HPLC as previously described in Chapter VI.

c. Isolation of lymphocytes

Separation of lymphocytes was performed as previously described in Chapter VII. The isolated lymphocytes were quantitated and frozen at -70°C until the time of analysis.

d. Determination of IMPDH activity

The activity of IMPDH in intact whole blood was measured using a procedure previously described in Chapter VII.

e. Measurement of nucleotide levels in lymphocytes

Patient lymphocytes were isolated from specimens collected throughout the 12 h dosing interval. Nucleotide levels were quantitated using a modification of a procedure previously described (23). Briefly, cells were

incubated for 30 min on ice with 500 μ L of 0.56 N perchloric acid (PCA). The insoluble material was removed by centrifugation and the supernatant collected and neutralized with 5 N KOH. The resulting precipitate was discarded after centrifugation and the resulting supernatant analyzed directly. The concentration of purine nucleotides was determined using a Hypersil-SAX HPLC column (4.6 mm id x 25 cm, Phenomenex, Torence, CA) at ambient temperature. Chromatographic separation was achieved using a nonlinear gradient as indicated: From 0-5 min 100% Buffer A (0.005 M phosphate buffer pH 2.8), 0% Buffer B (0.5 M phosphate buffer pH 3.8), from 5-15 min 0-10% Buffer B, 15-30 min 10-100% Buffer B. The system was re-equilibrated for 20 min in 100% Buffer A. The flow rate was 1.0 mL/min and detection was accomplished by UV absorbance at 254 nm. Quantitation was achieved using the peak height in relation to commercially available standards. An external standard curve was constructed for each analytical run, since no internal standard was available. All results were normalized to a lymphocyte count of 1.0×10^9 cells/L.

f. Pharmacokinetic analysis

Plasma concentration versus time data for MPA following administration of MMF were fitted to a non-compartmental model using PCNONLIN V 4.0 (Statistical Consultants Inc., Lexington, KY, USA). A total of 13 parameters are calculated by this program, including β half-life ($t_{1/2\beta}$), area under the curve (AUC), and mean residence time (MRT).

g. Statistics

Unless specified, all results are expressed as the mean $\pm$ SE. Statistical differences between groups were tested using a two way analysis of variance, paired or unpaired t-test, where appropriate, using SPSS statistical software (SPSS Inc., Chicago, IL). A p-value <0.05 was considered significant.

3. RESULTS

a. Normal distribution

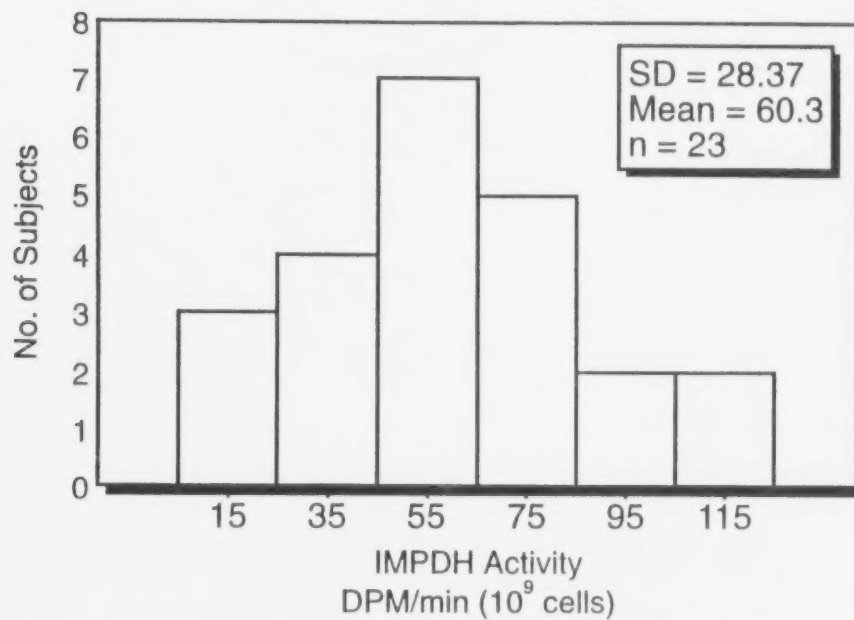
The IMPDH activity in whole blood from 22 normal subjects exhibits a gaussian distribution although a considerable variation in activity was observed with individual activity ranging from 21.8 to 121.6 dpm/min (Figure IX-1). This variability could most likely be attributed to biological rather than analytical variations since the coefficient of variation for the assay was $\leq 10\%$ was determined. The WBC count in the same patient population ranged from 3.5-7.5 $\times 10^9$ cells/L which is near the range of the WBC concentration curve (Chapter VII) that is linear.

b. Pharmacokinetic parameters

To investigate the relationship between plasma MPA concentrations and inhibition of IMPDH activity *in vivo*, 5 renal transplant patients were given an oral dose of MMF followed by serial collection of blood specimens at various times over a 12 hour period, with subsequent measurement of drug level and enzyme activity. The pharmacokinetic parameters obtained are summarized in

Figure IX-1.

Distribution of IMPDH activity in normal human volunteers.



IMPDH activity in 23 normal human subjects. IMPDH activity is expressed in dpm/min. The range of IMPDH activities was 21.8 to 121.6 dpm/min, and the WBC count ranged from 3.5 to 7.5×10^9 cell/L.

Table IX-1. The $t_{1/2}$ and AUC for this patient population was calculated to be approximately 6.1 h and 5.8 mg h/L respectively.

c. Pharmacodynamic analysis

The relationship between MPA concentration and IMPDH activity in a representative patient as well as for all patients combined are shown in Figures IX-2 and IX-3 respectively. A large variation among MMF treated patients (as indicated by the large SE) for both MPA concentration and IMPDH activity was observed as indicated in Figure IX-3. The peak concentrations of MPA achieved at 1 hour post-dose resulted in approximately 40% inhibition of IMPDH activity which is similar to that observed for the *in vitro* studies where approximately 50% inhibition was observed at similar concentrations (18). As the MPA concentration decreases throughout the dosing interval, there is a gradual restoration of enzyme activity. The inhibition of IMPDH activity is maintained for up to 8 hours post-dose, at which time the IMPDH activity begins to increase toward pre-dose levels. There was no statistical difference ($p < 0.05$) between the pre-dose and the 12 hours post dose IMPDH activity.

d. Comparison to azathioprine treated patients

No diurnal variation in IMPDH activity was noted in normal subjects throughout the dosing interval of 12 hours. This suggests that changes observed in the MMF treated group are drug induced. Since the patients used for this analysis were enrolled in a clinical trial comparing the efficacy of MMF to AZA, the PD responses of the AZA- and MMF-treated groups were

Table IX-1

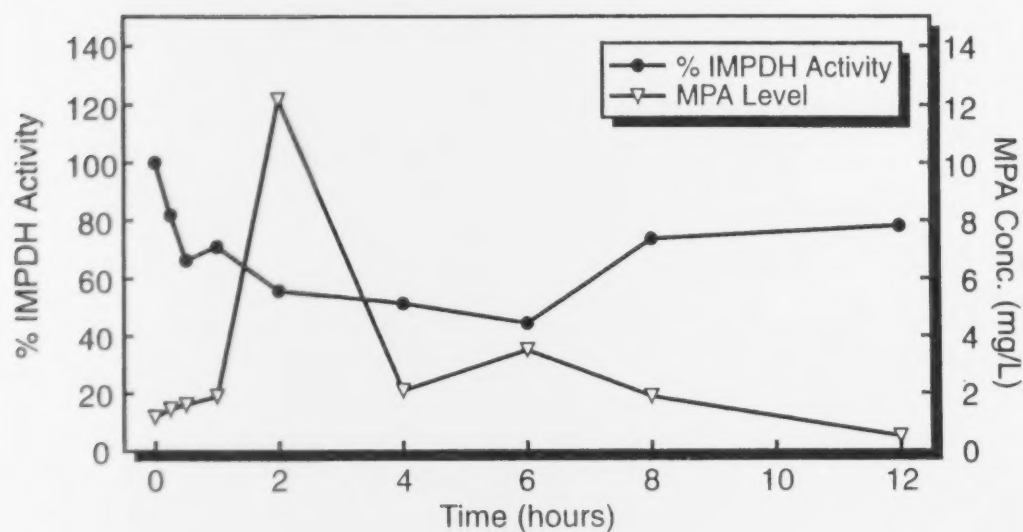
Pharmacokinetic parameters in renal transplant patients chronically treated with MMF following a single oral dose.

Patient	Tmax (h)	Cmax (mg/l)	t _{1/2} (h)	AUC (mg.h/L)	AUMC (mg/L)	MRT (h)
1	2.0	12.18	2.13	38.67	154.45	3.99
2	0.25	10.56	4.52	38.79	140.76	3.63
3	1.0	8.37	8.35	82.34	495.31	6.02
4	0.25	16.60	N/A ¹	41.75	620.09	N/A
5	1.0	21.96	9.47	89.48	1626.79	4.01
mean	0.90	13.93	6.12	58.21	607.48	4.41
SD	0.64	4.84	2.95	22.76	543.10	0.94

1 - unable to calculate

Figure IX-2.

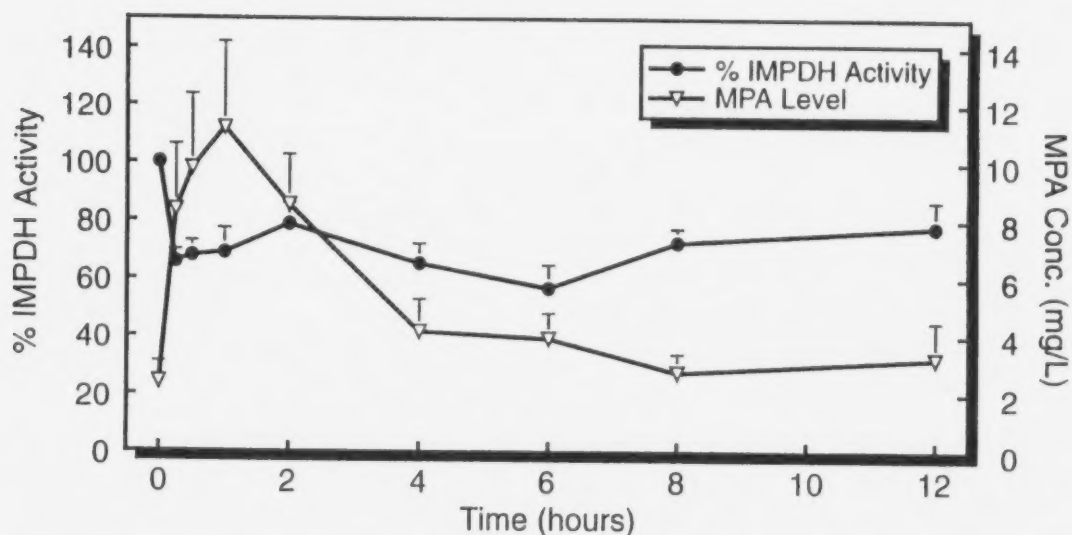
Pharmacokinetic/Pharmacodynamic profile of a representative renal transplant patient.



MPA concentration and IMPDH activity of a representative renal transplant recipient receiving a single oral dose of MMF. The patient had stable renal function and was at least 2 y post-transplant. Results are expressed as % change from time point 0. The patient's IMPDH activity was 54.8 dpm/min at time point 0, and the WBC count was 10.0×10^9 cells/L which did not change significantly over the sampling interval.

Figure IX-3.

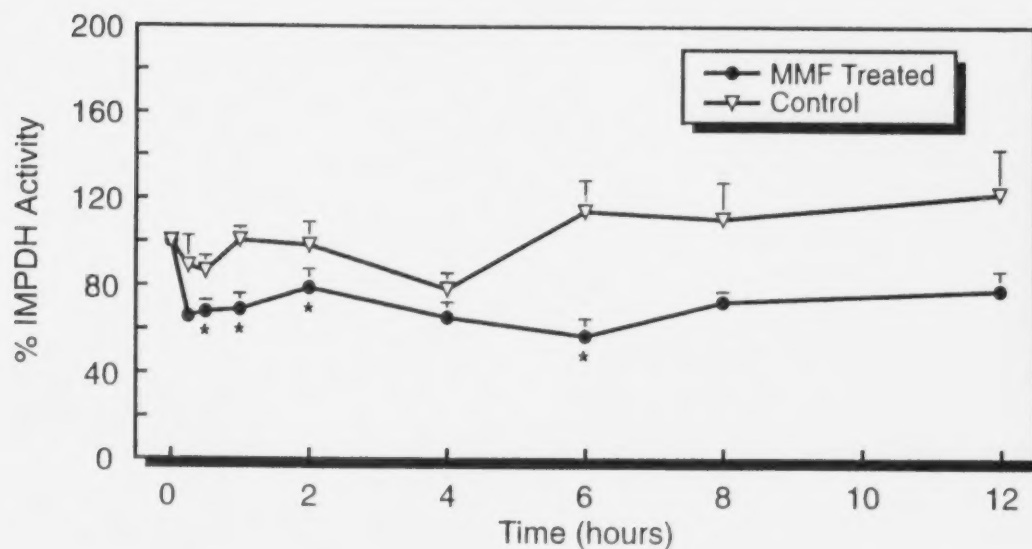
Mean Pharmacokinetic/Pharmacodynamic Profiles in renal transplant recipients.



Mean MPA concentration and IMPDH activity following administration of MMF throughout a 12 hour dosing interval. The patients had stable renal function and were at least 2 y post-transplant. Results are expressed as the mean % change from time point 0 $\pm$ SE (n = 5). The mean IMPDH activity was 49.8 ± 21.5 dpm/min at time point 0, and the patients WBC count ranged from 3.7 to 10.9 and did not change significantly over the sampling interval.

Figure IX-4.

Comparison of IMPDH activity in mycophenolate mofetil (MMF) and Azathioprine (AZA) treated renal transplant patients.



Mean IMPDH activity of MMF treated patients (n = 5) and AZA treated renal transplant patients (n = 7) through out a 12 h dosing interval. All patients had stable renal function at the time of the study and were at least 2 y post-transplant. Results are expressed as mean % change from time point 0 $\pm$ SE (for dpm/min values see page 186).

* $p < 0.05$

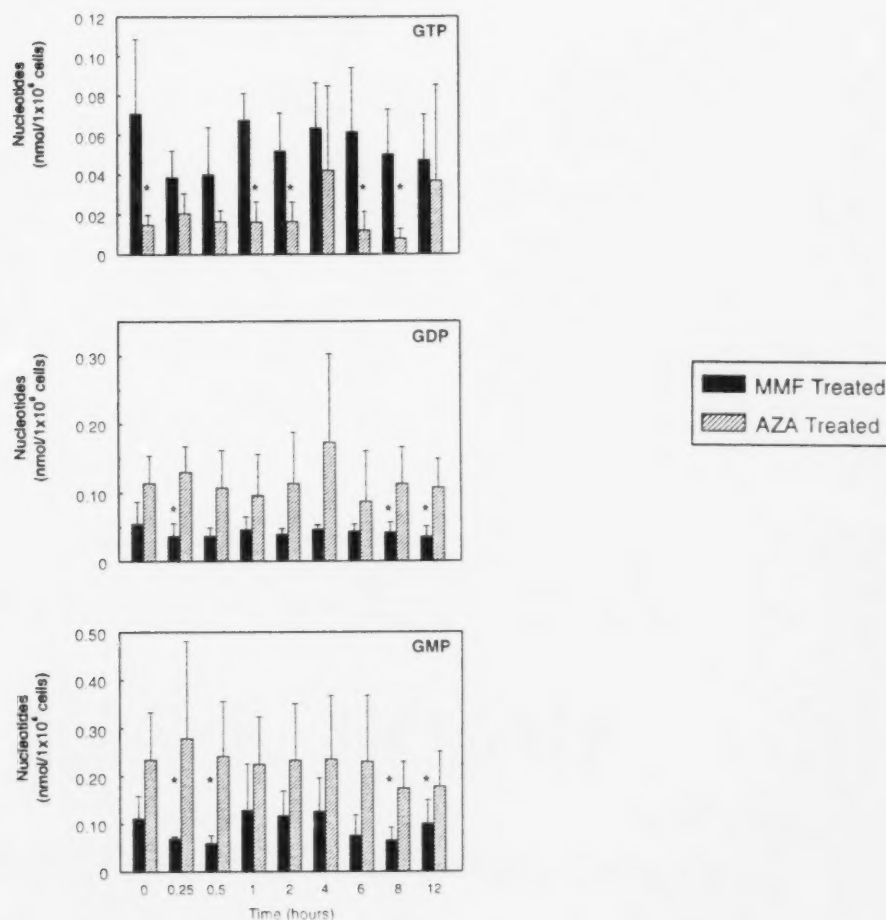
compared. The results are shown in Figure IX-4. There was no statistical difference ($p=0.22$) in the absolute activity of IMPDH at $t=0$ (pre-dose) in the MMF treated group (49.8 ± 21.5 dpm/min/ 10^9 lymphocytes) as compared to the AZA treated group (34.2 ± 13.6 dpm/min/ 10^9 lymphocytes) (Table IX-2). Within 1 hour of MMF administration there was a significant decrease in IMPDH activity in the MMF treated patient group as compared to the AZA treated group. This decrease was maintained throughout most of the dosing interval until approximately 8 hours post dose when the difference between the two groups was no longer significant ($p=0.06$), but the average IMPDH activity was still lower in MMF treated as compared to AZA.

e. Nucleotide levels

The lymphocytes at each time point post-dose were assayed for the purine nucleotides GMP, GDP, GTP, AMP, ADP, ATP and IMP. The results are shown in Figure IX-5. For each drug, there did not appear to be any significant change in the nucleotides throughout the dosing interval. In the MMF treated group the amount of GMP was statistically lower ($p \leq 0.05$) than the AZA group at some of the time points throughout the dosing interval. There was a trend to lower concentration of GDP in the MMF treated group than in the AZA group although statistical difference was only achieved at a few time points. The levels of GTP in the cells were found to be statistically higher in the MMF group than the AZA group. ADP, and ATP were not statistically different between the two groups at most of the time points. However, AMP levels in the MMF

Figure IX-5.

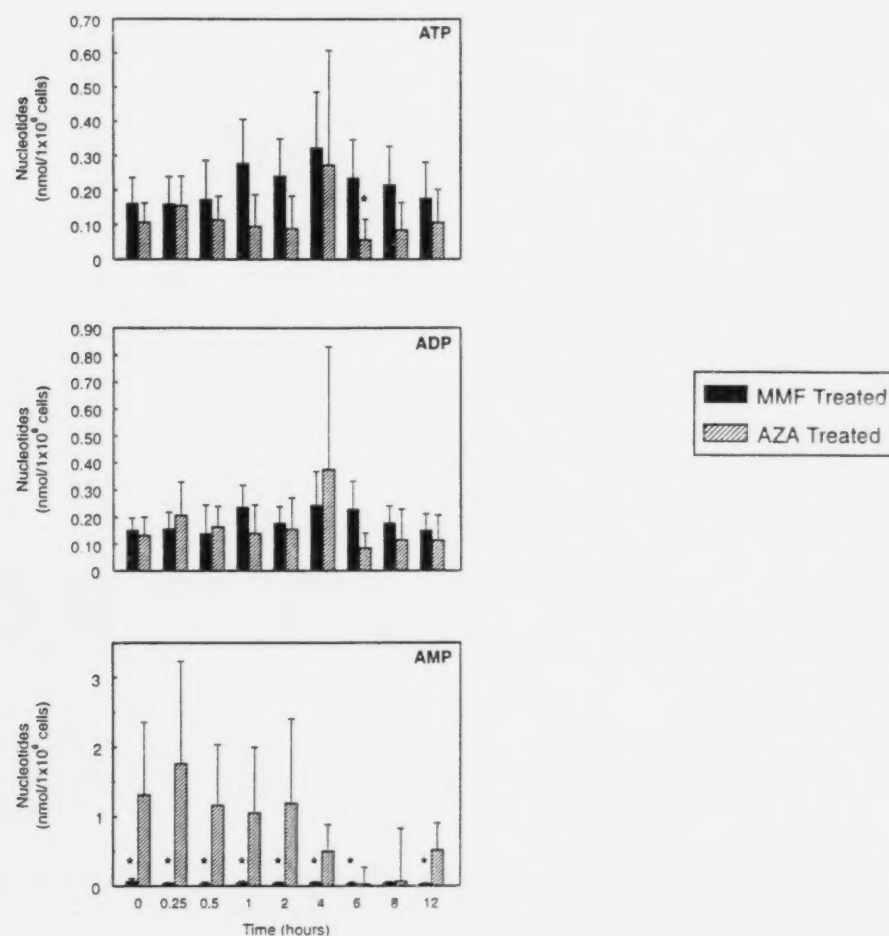
Concentration of guanine nucleotides in mycophenolate mofetil (MMF) and Azathioprine (AZA) treated patients



Mean concentration of guanine nucleotides in isolated lymphocytes for MMF treated patients (n = 5) and AZA treated patients (n = 7). Results are expressed as mean $\pm$ SD.

Figure IX-6.

Concentration of adenine nucleotides in mycophenolate mofetil (MMF) and Azathioprine (AZA) treated patients



Mean concentration of adenine nucleotides in isolated lymphocytes for MMF treated patients (n = 5) and AZA treated patients (n = 7). Results are expressed as mean $\pm$ SD.

treated group were lower than the AZA group. The intracellular levels of IMP in the AZA group were below the sensitivity of the assay. However, in the MMF treated group the intracellular IMP levels were approximately 0.15 pmol/ 10^9 cells.

4. DISCUSSION

Considerable inter-individual variation of IMPDH activity in normal subjects was noted, which could not be attributed to the analytical variability of the assay (intra-individual variation of IMPDH activity is $\leq 10\%$ and therefore within the analytical variation of the assay). Substantial variation among patients in absorption and clearance of MPA was observed in the pharmacokinetics of the drug in renal transplant recipients. The PK parameters for MPA reported in this study are similar to those previously reported (239). The large variation among the patients tested was not unexpected, as substantial pharmacokinetic variation among humans has been noted by others (233). This may have been due, in part, to the different dosages of MMF patients were receiving (1.0 or 1.5 g BID). However, we were not able to normalize the PK or PD parameters for dose due to the blinding of the latter. It has also been reported that following oral administration in humans, serum concentrations, as well as AUC, were significantly lower in the immediate post-transplant period as compared with day 20 post-transplantation (233). Unfortunately, pharmacokinetic parameters including volume of distribution and

clearance were not reported (233).

MPA, *in vitro*, has been shown to have immunosuppressive properties, mediated by inhibition of IMPDH activity, and leading to the depletion of guanine nucleotide pools (112,113,114,115). MPA induced inhibition of IMPDH activity *in vivo* was evaluated throughout a 12 h dosing interval. The results approximate an inverse relationship between MPA concentration and IMPDH activity. Maximal inhibition of IMPDH activity of approximately 40% occurs at approximately 0.5 h post-dose and C_{max} of MPA at 1 h post-dose. These results suggest that only a down-regulation, as compared to a complete inhibition of IMPDH activity, is required to result in immunosuppression. This down-regulation may be sufficient to alter the balance of purine and pyrimidine nucleotides (as indicated in Figure IX-5) resulting in an inhibition of DNA synthesis. The degree of inhibition of IMPDH activity is similar to that observed for cyclosporin on CN, which at therapeutic concentrations *in vivo* results in only a 50% inhibition in the activity of the latter (240,241,236). As the concentration of the drug decreases post-dose, there is a gradual increase in enzyme activity with little or no inhibition being observed at 12 h, indicating only a partial inhibition of IMPDH activity throughout the dosing interval. The sustained inhibition and gradual restoration of IMPDH activity are similar to what was observed in the rabbit (Chapter VIII), but are in contrast to what has been observed in the canine model. In dogs the peak concentration of drug occurs approximately 2 h post-dose and the maximum inhibition of IMPDH

activity is delayed until 6 h post-dose (Chapter VIII). The assessment of the pharmacodynamics of the drug may indicate which animal model most closely parallels the effect found in humans and therefore the appropriateness of the model for further studies with the drug. In individual patients, the maximum inhibition of IMPDH activity did not always parallel the time of peak MPA concentrations. *In vitro* studies have suggested that the free-MPA concentration may more accurately reflect the degree of drug induced inhibition of IMPDH activity than does the measurement of the total MPA (242). As drug concentrations increase post-dose the increase in free concentration may lag behind the increase in the total concentration as the plasma binding sites become saturable and as a new equilibrium is reached. The IMPDH activity may, therefore, more closely parallel the former rather than the latter drug concentrations. Studies investigating this *in vivo* are underway.

In the present study, renal transplant recipients were being dosed chronically every 12 h, the IMPDH activity returned to pre-dose levels prior to the next dose. This is similar to what is observed in rabbits, but in contrast to what is observed in dogs (Chapter VIII). The increase in enzyme activity seen in the dog was not observed (Chapter VIII). The degree of IMPDH inhibition in patients chronically treated with MMF was greater, in comparison, to the AZA treated group. A statistical decrease in the IMPDH activity seen after only 0.5 h post-dose and was maintained until approximately 2 h post-dose. As would be expected, this corresponds to the time interval when IMPDH activity begins

to increase toward pre-dose levels. At 12 hours post-dose the IMPDH activity was not statistically different ($p < 0.05$) from the pre-dose value. This suggests that in order to maintain inhibition of IMPDH activity throughout a dosing interval it would be appropriate to administer the second dose before the IMPDH activity returns to pre-dose levels. This indicates that dosing every 8 h could maintain inhibition of IMPDH activity. The absolute IMPDH activity 12 hours post-dose was similar in the MMF treated and the AZA treated groups which is most likely due to the transient effect of MMF on IMPDH activity.

The mechanism of AZA differs considerably from MPA. The latter drug selectively inhibits IMPDH. AZA is converted to the analogue 6-thioinosinic acid, which inhibits several enzymes of purine synthesis: IMPDH, PRPP-amidotransferase and adenylosuccinate synthase (243). The mechanism of both drugs can lead to a different effect on nucleotide pools. MPA administered at immunosuppressive doses was shown to significantly decrease the intracellular pool of GTP in lymphocytes and monocytes *in vitro* (5,28), which is in concordance with the *in vivo* results reported here. There is a statistical decrease in the intracellular concentration of GMP and a trend towards lower levels of GDP, when compared to the patients receiving AZA. The levels of GTP in the MMF group treated were not, however, lower than in the AZA group. The reason for this is unclear and requires further investigation. It has been suggested that the impairment of DNA synthesis by MPA is due to depletion of dGTP rather than GTP (65). There is also a significant decrease in

the amount of AMP in the MMF, as compared to the AZA-treated groups. Both MPA and AZA alter GTP pools. In the case of AZA, however, the mechanism of inhibition of proliferation appears to be completely unrelated to this effect on purine biosynthesis (244). It would be expected that MMF treatment would result in consistent and detectable alterations in the guanine nucleotide pool. Since AZA is also thought to alter nucleotide pools, significant differences between groups may not be apparent. However, in the MMF treated group there were detectable levels of IMP that were not apparent in the AZA treated group. Additionally, it has been determined that isolation of lymphocytes from blood reverses MPA induced inhibition of IMPDH, which is most likely due to loss of MPA from cells (Chapter VII). Although cells were processed as rapidly as possible, restoration of the guanine nucleotide pools in these cells could occur. There was no change in the nucleotide pools throughout the dosing interval, therefore, changes in nucleotide pools do not parallel those of drug concentrations. This suggests PD assessment by measurement of purine nucleotides levels in lymphocytes may not be informative.

X. CONCLUSIONS

Therapeutic drug monitoring is recommended when the drug in question has a narrow therapeutic index and is used chronically (245). It is difficult to establish a therapeutic range for CsA because there are no simple parameters for the assessment of the immunosuppressive effect. On the other hand, the lack of such criteria and the substantial intra-and inter-individual variation in CsA pharmacokinetics are strong arguments for drug monitoring to prevent over- or under-immunosuppression (246). A recent consensus conference outlined recommendations and guidelines for therapeutic drug monitoring of CsA (246). Since CsG was similar to CsA in metabolism and structure, it was generally assumed that the therapeutic monitoring protocols developed for CsA could be adapted for use with CsG (63,64). However, validation of this approach has not been reported.

An important factor to consider when monitoring CsG is the effect of the metabolites. The metabolites could contribute to both the immunosuppressive and the toxic effects, and both are dependent on concentration. The overall clinical significance of each CsG metabolite, expressed as a function of immunosuppressive activity, in vitro toxicity, and steady state concentration, is summarized in Table X-1. From this table, it can be concluded that the clinical significance of individual metabolites is markedly less than parent drug. Although individual metabolites are present in significant amounts, collectively, they contribute minimally to overall immunosuppression and drug-related side

Table X-1

CLINICAL SIGNIFICANCE OF CsG METABOLITES				
	RELATIVE STEADY STATE TROUGH CONCENTRATION $\mu\text{g/L}$ ^b	RELATIVE IMMUNOSUPPRESSIVE ACTIVITY ^a	RELATIVE TOXICITY ^d	CLINICAL SIGNIFICANCE INDEX
CsG	100	1	1.0	100
GM1	60	0.1	1.5	9.0
GM9	84	0.08	1.5	10.8
GM1c	24	0.01	< 1.0	< 0.24
GM4N	22	0.08	< 1.0	< 1.76
GM19	231	< 0.01	< 1.0	< 2.31
GM1c9	165	< 0.01	< 1.0	< 1.65
^a Clinical Significance Index (CS) = (Relative Steady State Trough Concentration) x (Immunosuppressive Potency) x (Toxicity) ^b chapter IV ^c Ref 56 ^d chapter V				

effects. This indicates that parent drug alone should be monitored.

Immunoassays originally developed for measurement of CsA have been adapted for measurement of CsG due to the significant cross-reactivity of assay antibodies for the latter. The cross-reactivities of CsG metabolites are comparable to CsA metabolites, however, the steady-state concentrations are significantly different. Based on the differences between the two drugs as described in this thesis, it can be concluded that problems can be encountered by utilizing the therapeutic monitoring protocols established for CsA. CsG should be treated as a new drug with the development of independent therapeutic monitoring guidelines. Although further investigation of the drug is not proceeding, the lessons learned from the experience with monitoring CsG can be applied to the development of other immunosuppressive drugs.

A number of approaches can be used to assess the appropriateness of the dosing regimen for immunosuppressive drugs. The first involves assessment of clinical response. This approach has its limitations since signs of rejection or toxicity may be difficult to recognize clinically. The second involves assessment of the PK properties of drug and relating various parameters (i.e., trough levels, AUC) to immunosuppressive efficacy or toxicity. This approach also has its limitations since the apparent concentrations of the drug measured may not reflect the pharmacologically active concentration due to cross-reactivity of inactive metabolites in the assays. In addition, therapeutic ranges for drugs have been difficult to establish due to dependency

on the type of transplant, time post transplant, and concomitant immunosuppressive drugs. The third, a pharmacodynamic approach, involves the measurement of the biological effect of the drug to monitor therapy and allow for dosage adjustments to optimize immunosuppression and minimize side-effects. This latter approach may have significant advantages over the measurement of drug concentrations especially in multiple drug therapy where the assessment of the appropriate therapeutic range may be difficult. Investigations using the immunosuppressive drug MPA were undertaken, since the measurement of its biological effect in lymphocytes is relatively straightforward and the drug will be routinely used in combination with other immunosuppressive agents, thus posing less difficulty in establishment of its therapeutic range. MMF was recently approved by the US food and Drug Administration for the prevention of rejection in renal transplant patients (177). The role of TDM as a therapeutic strategy has not been thoroughly evaluated.

The pharmacodynamic monitoring approach measures the total effect of a drug, including any contribution by metabolites. For MMF the measurement of IMPDH activity as described in this thesis provides a simple assay in whole blood. It was found that an inverse relationship between MPA concentration in plasma and IMPDH activity exists *in vitro* and *in vivo*. The extent of the MPA mediated inhibition of IMPDH activity *in vivo* is comparable to that *in vitro*.

The heterotopic heart transplant results in rabbits indicated that a

significant relationship exists between MPA concentration and dose of MMF. It was also determined that a significant correlation exists between MPA concentration and graft survival. This suggests that traditional TDM is more appropriate for the assessment of graft survival and as an indicator of the immune status of the patient. However, the measurement of the biological response may provide a useful adjunct to traditional TDM for the optimization of dosing regimes.

The measurement of IMPDH activity as described in this thesis measures the net enzyme activity. Upon stimulation there is an up-regulation of IMPDH type II isoenzyme. Secondly, the decrease of guanine nucleotides causes an induction of IMPDH at the transcriptional level. Therefore, in any patient treated with MMF there is, simultaneously, an inhibition of IMPDH by MPA and an induction of IMPDH due to the drugs effect. Since the assay, as described, cannot distinguish between inhibition and induction, a potential problem in the interpretation of the result occurs over time. To overcome this, one would need a type of control to compensate for any changes in the "total" IMPDH activity, for the reasons described above. This could be achieved by comparing the IMPDH activities in a patient sample in the presence of MPA and the same patient sample in which the MPA was removed. It was described in this thesis that MPA mediated inhibition of IMPDH activity could be removed by washing the cells. The measurement of IMPDH activity in the presence and absence of MPA may allow more accurate assessment of overall enzyme inhibition.

Pharmacodynamic monitoring has been applied to other immunosuppressive drugs including CsA. The immunosuppressive action is thought to be via a CsA-CyP complex that selectively inhibits calcineurin (CN). Inhibition of this phosphatase results in reduced transcription of IL-2 and other lymphokine genes. Pharmacodynamic approaches have, therefore, been based on the measurement of CN in peripheral blood lymphocytes. Synthetic phosphorylated polypeptides are used as substrates (247,240,235). The first report of pharmacodynamic monitoring of CsA by this method was in adult bone marrow transplant patients (247). The CN activities were analyzed with respect to CsA administration and concentration, and with the presence of GVHD. In all cases the CN activity was lower than that of normal subjects (247). The patients who had GVHD had lower CN activity, suggesting that CsA-resistant GVHD is not the result of inadequate suppression of CN activity (247). More recently in CsA-treated patients with therapeutic trough levels of CsA, CN activity was reduced by 50% as compared to controls (240). The induction of cytokine mRNA was not totally blocked, supporting the data indicating a lack of complete inhibition of CN activity (240). *In vitro*, however, the concentration required for 50% inhibition was about 10 fold less than *in vivo*. The difference is most likely due to the changes in the free fraction of CsA in tissue culture media as compared to biological fluids.

Another immunosuppressive agent under investigation for pharmacodynamic monitoring is AZA. It is metabolized into 6-MP in the liver

by the enzyme glutathione S-transferase. This conversion is dependent on the glutathione concentration. Further conversion of 6-MP occurs by HGPRT to 6-thioguanine, thought to be responsible for the immunosuppressive activity, and by thiopurine methyltransferase (TPMT) to 6-methylmercaptopurine (248). In renal transplant recipients, very low TPMT activity precluded the use of AZA because of severe myelotoxicity. More recently the erythrocyte TPMT activity was evaluated prospectively in renal transplant recipients with regard to efficacy of AZA (248). Those patients who exhibited an increase in TPMT activity from time of transplant experienced fewer episodes of acute rejection and, therefore, had better immunosuppression. It has been postulated that AZA-induced increase in TPMT reflected the appropriate conversion of AZA to 6-MP and, therefore, the immunosuppressive activity of the drug (248).

As the spectrum of immunosuppressive drugs available for use in transplantation increases in the future, establishment of therapeutic ranges for the drugs will become more difficult. Factors such as drug combinations, type of transplant, fraction of drug measured (total versus unbound), and the time post-transplant will have an impact on such ranges. This data shows that the measurement of the biological response to drugs may provide a viable alternative to traditional TDM by assessing the state of immunosuppression. Drugs, such as MMF, for which assessment of immunosuppression rather than the assessment of toxicity may be more amiable for pharmacodynamic monitoring (249,250).

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